

Anaerobic bacteria: an investigation of metabolic important enzymes

A novel type of oxygen reductase and enzymes of the tetrapyrrole biosynthesis

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Thesis Publications

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Dissertation Abstract

Desulfovibrio desulfuricans was the first species of a sulphate-reducing bacterium to be isolated, in 1895. Since that time, many questions were raised in the scientific community regarding the metabolic and ecological aspects of these bacteria. At present, there is still a myriad of open questions remaining to be answered to enlarge our knowledge of the metabolic pathways operative in these bacteria that have implications in the sulfur cycle, in biocorrosion, namely in sewers and in oil and gas systems, and in bioremediation of several toxic metals. The work presented in this dissertation aimed at contributing with new insights of enzymes involved in two different metabolic systems on *Desulfovibrio* species, namely enzymes that play a role in the response to oxidative stress and that are involved in the haem biosynthetic pathway.

Although for many years *Desulfovibrio* species have been considered strict anaerobes, several *Desulfovibrio* strains are found in environments where they are exposed to oxygen. In fact, these organisms contain several systems that enable the survival under oxidative conditions, but no aerobic growth was until now reported. In this dissertation it was shown, for the first time, that a *Desulfovibrio* species, *D. desulfuricans* ATCC 27774, has the ability to sustain growth when exposed to nearly atmospheric oxygen concentrations. Our studies suggest that *D. desulfuricans* ATCC 27774 utilizes oxygen for growth maintaining at the same time the nitrate dissimilatory pathway metabolism operative. Furthermore, enzymatic studies performed with protein fractions of *D. desulfuricans* ATCC 27774 cells exposed to 18 % oxygen, showed that proteins involved in scavenge of reactive oxygen species (ROS), namely the superoxide dismutase and catalase enzymes, have increased expression.

Analysis of the genomes of *Desulfovibrio* species, revealed that they contain genes coding for proteins that perform the reduction of oxygen to water, including the haem-copper membrane-bound oxygen reductases. A comprehensive amino acid sequence analysis of the haem-copper oxygen reductases encoded by the majority of *Desulfovibrio* sp. genomes, predicts the existence of a novel A2-type oxygen reductase, *ccaa3*, with an extra haem *c* binding motif (CxxCH) in subunit II. The biochemical characterization of a truncated form of *D. vulgaris* Hildenborough *ccaa3* subunit II confirmed the binding of the two *c*-type haems, with a His-Met haem-iron coordination. Furthermore, we showed that the *D. vulgaris* cytochrome *c*₅₅₃ donates electrons to the *ccaa3* oxygen reductase, revealing the first possible physiological electron donor to the haem-copper oxygen reductase in these organisms.

With the objective of clarifying the tetrapyrrole biosynthetic pathway in *D. vulgaris* Hildenborough, we performed the biochemical study of several enzymes involved in the generation of sirohydrochlorin, sirohaem and cobalt-sirohydrochlorin from aminolaevulinic acid, namely porphobilinogen synthase (HemB), porphobilinogen deaminase (HemC), uroporphyrinogen III synthase (HemD), uroporphyrinogen III methyltransferase (SUMT), precorrin-2 dehydrogenase (CysG^B) and sirohydrochlorin ferro- and cobalto-chelatases (CysG^B and CbiK, respectively).

These studies showed that *D. vulgaris* porphobilinogen synthase is a hexameric zinc-dependent enzyme and that the porphobilinogen deaminase contains a dipyrromethane-cofactor. The gene for *D. vulgaris* uroporphyrinogen III synthase is fused with the gene encoding uroporphyrinogen III methyltransferase. Both activities were demonstrated in the complete protein, and two separate domains of the protein, generated by molecular genetic techniques, exhibit the correspondent activities. A bi-funcional precorrin-2 dehydrogenase/

ferrochelatase encoded in the genome of *D. vulgaris* was demonstrated to contain only the dehydrogenase activity, being simply capable of synthesising sirohydrochlorin rather than sirohaem. *D. vulgaris* contains two CbiK cobaltochelataes (CbiK^P and CbiK^C) that were shown in the present study, to perform *in vitro* cobalt and iron chelation into sirohydrochlorin. CbiK^P has a signal peptide that exports the protein to the periplasm, whereas CbiK^C is located in the cytoplasm. Expression of CbiK^C and a form of CbiK^P lacking the signal peptide, from a multicopy vector, restored the wild type phenotype of an *E. coli* mutant strain deficient in sirohydrochlorin ferrochelatase activity, showing that they can act in the *in vivo* synthesis of sirohaem in *E. coli*.

D. vulgaris CbiK^P is a tetrameric protein that contains a *b*-type haem, which is so far a unique feature within the chelatase-family of enzymes. We have demonstrated that haem *b* of CbiK^P is not involved in the chelatase activity and the three-dimensional structure of CbiK^P revealed that the haem is located in between two monomers and coordinated by two histidines, namely His96 of each monomer. Co-crystallisation of CbiK^P with cobalt showed three amino acid residues involved in cobalt binding: His154, Glu184 and His216. Moreover, comparison of the structure with and without cobalt shows that His154, a strictly conserved amino acid, is the only residue that undergoes a significant alteration, most probably to allow the coordination of the cobalt ion.

It was also shown that *D. vulgaris* Hildenborough synthesises vitamin B₁₂ at the cytoplasm level, being CbiK^C the most likely enzyme to be involved in this process. Although the function of CbiK^P remains unclear, our analysis of the *D. vulgaris* genome together with biochemical and genetic studies of cobaltochelataes from other organisms, suggest a role of *D. vulgaris* CbiK^P in haem/iron transport.

The presence in *D. vulgaris* of a bi-functional uroporphyrinogen III synthase/methyltransferase suggests that uroporphyrinogen III is not released as an intermediate. Therefore, uroporphyrinogen III cannot be acted upon by the first enzyme of the haem biosynthetic branch of the tetrapyrrole pathway. This event is consistent with a proposal made in the 1990's of an alternative pathway being operative in *Desulfovibrio*, in which precorrin-2 would be the intermediate in the haem synthesis pathway. However, the enzymes necessary to convert precorrin-2 to haem are not known, given that *Desulfovibrio* genomes lack the genes encoding the canonical haem synthesizing enzymes. Since our analysis of the genome of *D. vulgaris* show the presence of two genes encoding NirJ and NirD, two proteins involved in the synthesis of haem d_1 , which is made via precorrin-2, we proposed that in *Desulfovibrio* the enzymes of the d_1 biogenesis may be involved in the conversion of precorrin-2, sirohydrochlorin or sirohaem, into haem. The proposed pathway of haem biosynthesis is discussed in the final chapter of this dissertation.

Resumo da dissertação

Desulfovibrio desulfuricans foi a primeira espécie do grupo das bactérias redutoras de sulfato a ser isolada, em 1895. Desde essa altura, muitas questões sobre o metabolismo e ecologia destas bactérias foram discutidas na comunidade científica. Actualmente, existem ainda inúmeras questões em aberto que precisam de ser exploradas, de forma a alargar o nosso conhecimento das vias metabólicas operativas nestes organismos, que têm implicações directas no ciclo do enxofre, biocorrosão em esgotos e na indústria do petróleo e do gás e na bioremediação de vários metais tóxicos. O trabalho apresentado nesta dissertação teve como objectivo contribuir para o conhecimento das enzimas envolvidas em dois sistemas metabólicos diferentes de *Desulfovibrio*, nomeadamente enzimas que desempenham funções na resposta ao stress oxidativo e enzimas envolvidas na via biosintética dos hemos.

Apesar das espécies de *Desulfovibrio* serem consideradas desde há muitos anos como anaeróbias estritas, várias estirpes de *Desulfovibrio* vivem em habitats expostos a oxigénio. De facto, estes microorganismos possuem vários sistemas que permitem a sua sobrevivência em condições oxidativas mas o seu crescimento aeróbio nunca foi reportado. Nesta dissertação é apresentada pela primeira vez uma espécie de *Desulfovibrio*, *D. desulfuricans* ATCC 27774, que tem a capacidade de crescer quando exposta a concentrações atmosféricas de oxigénio. Os nossos resultados sugerem que *D. desulfuricans* ATCC 27774 utiliza oxigénio para crescer, retendo ao mesmo tempo a capacidade de respiração de nitrato. Estudos realizados em fracções proteicas de células de *D. desulfuricans* ATCC 27774 submetidas a 18 % de oxigénio, mostram um aumento da expressão de proteínas envolvidas na destoxificação de espécies reactivas de oxigénio, nomeadamente a dismutase de superóxido e a catalase.

A análise dos genomas das espécies de *Desulfovibrio* revelou que estes organismos possuem genes que codificam para proteínas que fazem a redução do oxigénio a água, incluindo redutases de oxigénio hemo-cobre. A análise das sequências de aminoácidos das redutases de oxigénio hemo-cobre, codificadas nos genomas da maioria das espécies de *Desulfovibrio*, prevê a existência de uma nova enzima da família A2, a *cca3*, que contém na sua subunidade II um motivo extra de ligação a um hemo *c* (CxxCH). A caracterização bioquímica de uma forma truncada da subunidade II da enzima *cca3* de *D. vulgaris* Hildenborough confirmou a ligação de dois hemos do tipo *c*, com uma coordenação His-Met ao ferro do hemo. Neste trabalho foi ainda mostrado que o citocromo *c₅₅₃* dá electrões à *cca3*, revelando-se o primeiro possível dador de electrões fisiológico para a redutase de oxigénio hemo-cobre nestes organismos.

Com o objectivo de elucidar a via biosintética dos tetrapirroles em *D. vulgaris* Hildenborough, foi realizado o estudo bioquímico de várias enzimas envolvidas na síntese de sirohidroclorina, sirohemo e cobalto-sirohidroclorina, a partir de ácido aminolevulínico, nomeadamente porfobilinogénio sintase (HemB), porfobilinogénio deaminase (HemC), uroporfirinogénio III sintase (HemD), uroporfirinogénio III metiltransferase (SUMT), precorrina-2 desidrogenase (CysG^B), sirohidroclorina ferroquelatase (CysG^B) e sirohidroclorina cobalto-quelatase (CbiK).

Estes estudos mostraram que a proteína porfobilinogénio sintase de *D. vulgaris* é uma enzima hexamérica, cuja actividade é dependente de zinco, e que a porfobilinogénio deaminase contém um dipirrometano como cofactor. O gene que codifica para a uroporfirinogénio III sintase em *D. vulgaris* encontra-se fundido com o gene da uroporfirinogénio III metil-transferase. Ambas as actividades foram demonstradas na proteína completa, e os dois domínios da proteína, que foram separados utilizando técnicas de genética molecular, exibem as actividades

correspondentes. A proteína bi-funcional precorrina-2 desidrogenase/ferroquelatase, codificada no genoma de *D. vulgaris*, foi demonstrada possuir apenas a actividade de desidrogenase, sendo capaz de sintetizar sirohidroclorina e não sirohemo, como proposto. *D. vulgaris* tem duas cobaltoquelatases (CbiK^P e CbiK^C) e neste estudo foi demonstrado que ambas fazem a inserção *in vitro* de cobalto e ferro na sirohidroclorina. A CbiK^P possui um sinal peptídico que a transporta para o periplasma, enquanto que a proteína CbiK^C é citoplasmática. Quando expressas a partir de um vector de cópia múltipla, numa estirpe mutante de *E. coli* que é deficiente em actividade de ferrochelataase de sirohidroclorina, as duas quelatases (CbiK^C e uma forma truncada da CbiK^P, em que o sinal peptídico foi removido) são capazes de restabelecer o fenótipo da estirpe de *E. coli* selvagem, demonstrando a sua capacidade de participar na síntese *in vivo* do sirohemo.

A CbiK^P de *D. vulgaris* é uma proteína tetramérica que contém um hemo do tipo *b*, uma característica que é até agora única na família das quelatases. Os nossos estudos demonstraram ainda que o hemo *b* da CbiK^P não participa na actividade de ferro- ou cobalto-quelataase e a estrutura tridimensional da CbiK^P revelou que o hemo está localizado entre dois monómeros sendo coordenado por duas histidinas, uma de cada monómero (His96). A co-cristalização da proteína CbiK^P com cobalto mostrou que existem três aminoácidos envolvidos na ligação ao cobalto: His154, Glu184 e His216. A comparação da estrutura da CbiK^P com e sem cobalto permitiu verificar que a His154, um aminoácido estritamente conservado, é o único resíduo que sofre uma alteração posicional significativa, provavelmente para permitir a coordenação do ião cobalto.

Foi também mostrado que *D. vulgaris* Hildenborough sintetiza vitamina B₁₂ ao nível do citoplasma, e que muito possivelmente a proteína CbiK^C está envolvida neste processo. Apesar da função da

CbiK^P ser ainda desconhecida, a nossa análise do genoma de *D. vulgaris* em conjunto com estudos bioquímicos e genéticos efectuados com cobaltoquelatases de outros organismos, sugere o envolvimento da CbiK^P de *D. vulgaris* no transporte de hemo ou/e ferro.

A presença em *D. vulgaris* da proteína bi-funcional uroporfirinogénio III sintase/metil-transferase, sugere que o uroporfirinogénio III não é libertado como intermediário. Desta forma, este composto não pode servir de substrato à primeira enzima que actua no ramo biossintético do hemo da via metabólica dos tetrapirroles. Este facto é consistente com uma proposta feita nos anos 90, que sugere a existência de uma via alternativa em *Desulfovibrio*, na qual a precorrina-2 seria um intermediário na via biosintética dos hemoss. No entanto, as enzimas necessárias para converter a precorrina-2 em hemo não são conhecidas, uma vez que os genomas de *Desulfovibrio* não codificam para as enzimas clássicas que participam na via biossintética do hemo. Uma vez que a nossa análise do genoma de *D. vulgaris* mostra a existência de dois genes que codificam para duas proteínas, a NirJ e a NirD, que estão envolvidas na síntese do hemo *dh*, que ocorre via a precorrina-2, nós propusemos que em *Desulfovibrio* as enzimas da biogénese do hemo *dh* estão envolvidas na conversão da precorrina-2, da sirohidroclorina ou do sirohemo, em hemo. A via proposta para a biossíntese dos hemoss em *Desulfovibrio* é discutida no capítulo final desta tese.

Abbreviations

Δ	Deletion
Å	Angstrom
Acetate	—CH ₂ COOH
ADP	Adenosine diphosphate
ALA	δ -Aminoleaevulinic acid
ATCC	American type culture collection
ATP	Adenosine triphosphate
bp	Base pair
CoA	Coenzyme A
CO	Carbon monoxide
CO ₂	Carbon dioxide
Da	Dalton
DCIP	2,6-Dichlorophenolindophenol
DNA	Deoxyribonucleic acid
e-	Electron
E_m	Redox potential
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
Fe-S	Iron-sulphur cluster
FMN	Flavin mononucleotide
g	EPR g -factor
H ₂	Hydrogen
H ⁺	Proton
HMB	Hydroxymethylbilane
HPLC	High performance liquid chromatography
IPTG	Isopropyl- β -D-thiogalactopyranoside
LB	Luria Bertani
M	Molar
Mb	Mega base pair
Methyl	—CH ₃
NAD	β -Nicotinamide adenine dinucleotide, oxidized form
NADH	β -Nicotinamide adenine dinucleotide, reduced form
NADP	β -Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	β -Nicotinamide adenine dinucleotide phosphate, reduced form
NMR	Nuclear magnetic resonance
PBG	Porphobilinogen
PCR	Polymerase chain reaction
Pi	Inorganic phosphate
Propionate	—CH ₂ CH ₂ COOH
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAM/SAH	S-adenosyl-methionine/ S-adenosyl-homocysteine
SO ₄ ²⁻	Sulphate
SUMT	S-adenosyl-methionine:uroporphyrinogen III methyltransferase
H ₂ S	Sulphide/hydrogen sulphide
sp.	Species
SRB	Sulphate reducing bacteria
TMPD	N,N, N',N'-Tetramethyl- <i>p</i> -phenylene diamine
tRNA	Transfer ribonucleic acid
UV	Ultraviolet
Vinyl	—CH=CH ₂
Vis	Visible
wt	Wild type

Latin abbreviations

<i>i.e.</i>	<i>id est</i> , that is to say
<i>e.g.</i>	<i>exempli gratia</i> , for example
<i>et al.</i>	<i>et alia</i> , and other people

Aminoacids

G	Gly	Glycine
A	Ala	Alanine
L	Leu	Leucine
M	Met	Methionine
F	Phe	Phenylalanine
W	Trp	Tryptophan
K	Lys	Lysine
Q	Gln	Glutamine
E	Glu	Glutamic Acid
S	Ser	Serine
P	Pro	Proline
V	Val	Valine
I	Ile	Isoleucine
C	Cys	Cysteine
Y	Tyr	Tyrosine
H	His	Histidine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartic Acid
T	Thr	Threonine

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Discussion

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1.1 - Sulphate Reducing Bacteria

1.1.1 - An overview on sulphate reducing bacteria

Sulphate reducing bacteria (SRB) are a large and heterogeneous group of prokaryotic microorganisms that perform dissimilatory sulphate reduction, *i.e.*, they are able to couple the anaerobic oxidation of organic compounds or molecular hydrogen to the reduction of sulphate to hydrogen sulphide in order to obtain energy for growth (1). Since this is a very ancient process, some of the oldest life forms on Earth probably belong to this group of microorganisms (2). The dissimilatory sulphate reduction product, hydrogen sulphide, has a typical smell and in the presence of iron minerals forms black precipitates of ferrous sulphide, which makes easy the recognition of SRB habitats (1). In fact, SRB are widespread in nature and mainly found in sulphate-rich anoxic habitats such as soil, marine and fresh waters and sediments as well as in the oxic-anoxic interfaces of all these biotopes and in the gut of many animals, including humans. Although named after a single electron acceptor, SRB are metabolic versatile and therefore able to use other organic or inorganic compounds as terminal electron acceptors, such as nitrate, nitrite, carbon dioxide, iron(III), fumarate, elemental sulphur and other sulphur species (3, 4).

This group of microorganisms has a significant environmental impact in several aspects: i) they participate in the carbon and sulphur cycle by recycling sulphur compounds in the degradation of organic matter; ii) SRB are involved in bioremediation, being able to reduce several toxic metals that constitute an environmental pollution problem with great impact in public health and economy (5). Apart from the beneficial point of view, SRB are a major problem in oil industry due to the production of sulphide, which in high concentrations leads to corrosion of the pipelines and equipments used in the oil processing. More recently, it was showed that SRB communities are also present in rock art caves and might contribute to the

biodeterioration of paleolithic paintings (6). As inhabitants of the animal and human intestine, SRB have health relevance and some studies led to the hypothesis that SRB may play a role in inflammatory bowel diseases (7).

1.1.2 - Historical perspective

It was in 1895 that the Dutch microbiologist Martinus Beijerinck (Figure I-1.1) described the first sulphate reducer, *Spirillum desulfuricans* (Figure I-1.2) (8). Beijerinck isolated this bacterium from a Dutch city canal in Delft and described it as the cause of contamination of the city sewages in the summer due to the production of hydrogen sulphide. Since he observed that *Spirillum desulfuricans* was difficult to growth in the absence of aerobic bacteria, that were required to consume the oxygen in the culture, the bacterium was classified as strict anaerobe (9, 10).

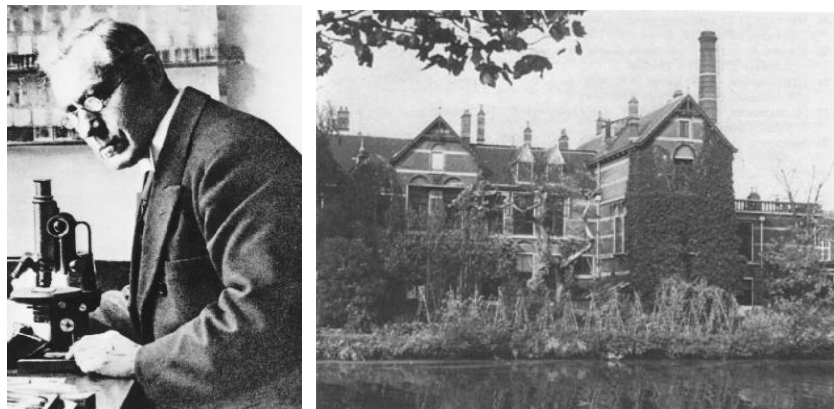


Figure I-1.1 - Left: Martinus Beijerinck (1851-1931), Professor of microbiology at the Technical School in Delft (Netherlands) which isolated the first sulphate reducing bacterium. Right: Beijerinck's Laboratory for Microbiology in Delft.

In 1930, Baars studied *Spirillum desulfuricans* and demonstrated that this bacterium was able to oxidize lactate and ethanol to acetate. At this

time, this bacterium was named *Vibrio desulfuricans* and later was renamed *Desulfovibrio desulfuricans*, by Kluyver and van Niel (11).

The discovery that sulphate reducers could oxidize H_2 occurred in 1931 and was between 1950 and 1960 that the first steps on sulphate-reducer biochemistry were made, leading to important discoveries such as the existence of cytochromes, the pigments that were only known to be associated with aerobic respiration, and the presence of a green enzyme, named desulfoviridin, further recognized as a sulphite-reductase (1).

Following this period of time, several areas of study emerged allowing a

better understanding of these bacteria. These included the taxonomic classification of several sulphate reducers, the exploration of alternate electron donors and acceptors for SRB growth, and the biochemical and functional characterization of several enzymes present in these microorganisms.

In the 1960s, the taxonomic classification of SRB was essentially based in cell morphology and ability to form spores. Although SRB are a group of prokaryotes with different types of cell morphology (*e.g.* cocci, oval, rods, curved (vibrioid) types, cell aggregates and filaments), these bacteria were first classified in two genera on the basis of morphology: the vibrio-shaped and nonspore-forming genera *Desulfovibrio* and the rod-shaped spore-forming genera *Desulfotomaculum* (12). This phylogenetic classification was soon altered considering additional features such as metabolism and biochemical or chemical markers. However, only in the late 1980s, a more



Figure I-1.2 - Painting of *Vibrio desulfuricans* (*Desulfovibrio desulfuricans*) by the sister of Martinus Beijerinck (9).

accurate classification of SRB was achieved based on the 16S rRNA sequences of the several strains (13), and the present phylogenetic classification of SRB takes in account several taxonomic markers such as the cell morphology, the 16S rRNA, the nutritional characteristics, the presence of special pigments and the DNA guanine+cytosine (G+C) content. In addition, recent studies on lateral gene transfer events of gene fragments encoding enzymes of the sulphate respiration pathway (*e.g.* dissimilatory adenosine-5'-phosphosulphate (APS) reductase (ApsAB) and the dissimilatory sulphide reductase (DsrAB)) between the several species, provided a more restrict evolutionary classification of SRB (14, 15).

Although sulphate reducers have been studied for more than one century, only in the last few years a better understanding of their life and the metabolic implications in environment and human health has been possible as a result of studies at the genetic, biochemical and molecular level (see (16) for review). Still, there are many questions that remain to be addressed to fully understand the importance of these organisms.

1.2 - *Desulfovibrio*

Desulfovibrio, which belongs to the class of δ -proteobacteria is one of the best studied genus of sulphate reducing bacteria. Several *Desulfovibrio* species are found in aquatic habitats (marine and fresh waters), soil and sediments, oil and natural gas wells, sewages and in the gut of animals and humans.

Cells of *Desulfovibrio* present Gram-negative staining, curved or rod morphology and are often motile (Figure I-1.3). They typically contain high mol % and, in general, contain the sulphite reductase desulfoviridin and the tetrahaem

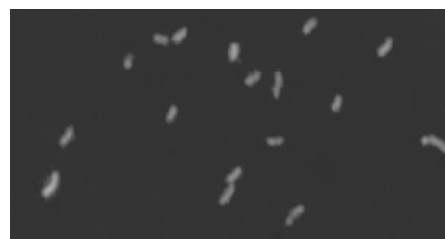


Figure I-1.3 - *Desulfovibrio desulfuricans* ATCC 27774 curved (vibrio) shaped cells, observed under optical microscopy (1000x).

cytochrome *c*₃. The optimal growth temperature of *Desulfovibrio* strains is between 30-38 °C (17, 18).

Advances in genome sequencing have contributed for a better understanding of the general metabolism of *Desulfovibrio* species (19). Presently, five strains of *Desulfovibrio* have its genome completely sequenced: *D. vulgaris* str. Hildenborough (20), *D. vulgaris* str. PD4, *D. vulgaris* str. Myasaki, *D. desulfuricans* str. G20 and *D. desulfuricans* str. ATCC 27774 (21). Genome sequencing of other *Desulfovibrio* strains are currently in progress, namely for *D. salexigens* str. DSM 2638 (21), *D. magneticus* str. RS-1 (22) and *D. piger* str. ATCC 29098 (23). The mol % of G+C in these *Desulfovibrio* genomes varies between 47-67 % and the size of the genomes contains between 3.5-4.2 Mb, being the genome of *D. desulfuricans* ATCC 27774 the smallest, with approximately 2.9 Mb.

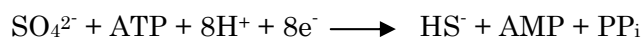
1.2.1 - Energy metabolism of *Desulfovibrio*

As previously mentioned, *Desulfovibrio* sp. gain energy by performing dissimilatory sulphate reduction, a process where sulphate is reduced to sulphide, coupled with the oxidation of H₂ or organic substrates. Besides sulphate, several other electron acceptors are used, such as sulphite, thiosulphate, sulphur, nitrate, elemental Fe(III), CO₂ and fumarate. Growth of *Desulfovibrio* with fumarate as electron acceptor involves a quinol:fumarate oxidoreductase (QFR) that reduces fumarate to succinate. The latter can also catalyse the reverse reaction of succinate oxidation although this reaction is normally catalysed by succinate:quinone oxidoreductase (SQR) (24). The most common organic substrates utilized by *Desulfovibrio* are lactate, pyruvate and ethanol. These substrates are incompletely oxidized to acetate and carbon dioxide due to the apparent lack of a mechanism for acetyl-coenzyme A (CoA) oxidation. Consequently members of this genus are called “incomplete oxidizers”. *Desulfovibrio* sp. are also able to use ethanol, glycerol, amino acids (glycine, alanine and

serine) and sugars (fructose) as electron donors. Growth in the absence of external electron donor is also possible by fermentation of sulphite and thiosulphate to sulphate and sulphide. This process is known as disproportionation. Fumarate and malate can also be disproportionated to yield succinate (1, 25).

Sulphate reduction

The reduction of sulphate occurs in the cytoplasm near the inner side of the cytoplasmic membrane by an overall reaction that involves eight electrons and requires ATP (1):



The energy loss is compensated by proton-gradient coupled to phosphorylation of ADP to ATP (1). External sulphate is initially transported across the membrane, to the cytoplasm, by an ion-gradient. This uptake is achieved either by simultaneous transport of sulphate with protons, in the case of *D. desulfuricans* (26) or with sodium ions, as in *D. salexigens* (27). Upon entering the cytoplasm, sulphate, which is thermodynamically stable and by itself is not a suitable electron acceptor, has to be activated (3). The activation of sulphate to adenosine 5'-phosphosulphate (APS) is made at the expense of ATP in a reaction catalysed by ATP sulfurylase (Figure I-1.4). The next steps of the pathway are the reduction of APS to bisulphite and adenosine-5'-phosphate (AMP), catalysed by APS reductase, and the reduction of bisulphite to sulphide, by the enzyme bisulphide reductase, which in *Desulfovibrio* is known as desulfovibridin (1, 3). The mechanism for the latter reaction is still not completely clear and two different pathways have been proposed (Figure I-1.4). In one of the pathways (trithionate pathway), the reduction proceeds via two intermediates, thiosulphate and trithionate, formed by trithionate and thiosulphate reductases (28) whereas in the other pathway, bisulphite is reduced to sulphide in one six electron step (29). However, recent results

have shown that growth of *D. vulgaris* on sulphate and H₂ was not impaired when thiosulphate reduction was abolished, thus it was proposed that under these growth conditions, APS and HSO₃⁻ are the only intermediates (30).

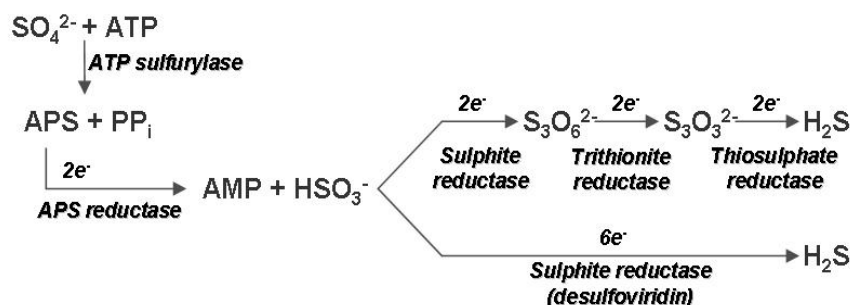


Figure I-1.4 - Proposed pathways of dissimilatory sulphate reduction (adapted from (25) and (17)).

Nitrate and nitrite reduction

D. desulfuricans ATCC 27774 is capable of using nitrite and/or nitrate as electron acceptors. Nitrate is reduced to ammonia in a dissimilatory pathway that involves the nitrate reductase and nitrite reductase enzymes. The first step is the conversion of nitrate to nitrite, performed by the enzyme nitrate reductase. *D. desulfuricans* ATCC 27774 has a dissimilatory periplasmic nitrate reductase (Nap) which contains one molybdenum cofactor in the active site and a [4Fe-4S] cluster. The crystal structure of *D. desulfuricans* Nap was the first to be solved for a nitrate reductase (31). The six electron reduction of nitrite to ammonia is performed by nitrite reductase (NiR) enzymes. Two different types of NiRs are known: the cytoplasmic sirohaem NiRs, proposed to be involved in the detoxification of nitrite, and the membrane bound or periplasmic cytochromes *c* NiRs, which are involved in the dissimilatory nitrate pathway. The dissimilatory cytochrome *c* nitrite reductase of *D. desulfuricans* ATCC 27774 is a membrane-bound protein composed by two subunits, the pentahaem catalytic subunit NrfA, located at the periplasmic

side, and the transmembrane tetrahaem subunit, NrfH (32). *D. vulgaris* Hildenborough is not able to reduce nitrate but it can reduce nitrite to ammonium through a constitutive cytochrome *c* NiR, in a reaction that does not sustain cell growth (33).

Hydrogen oxidation

Hydrogen can be used by *Desulfovibrio* as the sole energy source, with acetate and carbon dioxide as carbon sources. Hydrogen is oxidized by enzymes called hydrogenases that can be cytoplasmic, periplasmic or membrane-associated. Three types of hydrogenases are distinguished in *Desulfovibrio*: the [Fe], [NiFe] and [NiFeSe] hydrogenases. Besides metal composition, these enzymes have differences concerning molecular mass, catalytic activity and sensitivity for CO, NO, nitrite and acetylene (34). The [Fe] hydrogenase of *D. desulfuricans* contains two ferredoxin-type [4Fe-4S]^{2+/1+} clusters and a [4Fe-4S] cluster bridged to a binuclear active site Fe center that is involved in H₂ activation (35). The first solved three-dimensional structure of a hydrogenase was the periplasmic [NiFe] hydrogenase from *D. gigas* which contains besides the nickel-iron binuclear active site, two [4Fe-4S]^{2+/1+} clusters and one [3Fe-4S]^{1+/0} cluster (36). The structure of the periplasmic [NiFeSe] hydrogenases from *Desulfomicrobium baculatum* showed that the enzyme contains three [4Fe-4S]^{2+/1+} clusters and that selenium is present in the form of a selenium-cysteine residue bound to the nickel-iron binuclear center (37).

In order to get energy for sulphate reduction, the periplasmic hydrogenases have to oxidize four H₂ and the eight protons and electrons released are transferred via ATP synthase and transmembrane protein complexes, respectively, to the cytoplasm. The electrons are transferred to the transmembrane protein redox complexes via type I tetrahaem cytochrome *c*₃ (TpIc₃) (the physiological electron acceptor of the periplasmic hydrogenase) (see (38) for a review). The charge separation leads to the

reduction of sulphate and production of ATP by ATP synthase as consequence of the generation of a membrane potential. This process is called vectorial electron transport (39). From the eight protons available, one can be transported with sulphate to the cytoplasm (26) leaving only seven protons for ATP synthase. Consequently, $2\frac{1}{3}$ mol of ATP are formed, two of which are used for sulphate reduction, leaving $\frac{1}{3}$ ATP mol as net energy for cell synthesis.

Lactate, pyruvate and formate oxidation

Lactate is the “classical” substrate for most *Desulfovibrio* sp.. Lactate and pyruvate are incompletely oxidized to acetate and carbon dioxide when they serve as electron donors for sulphate reduction. The oxidation of lactate to pyruvate is catalysed by a membrane bound NAD(P)⁺-independent lactate dehydrogenase (40). Pyruvate is oxidized to acetate and carbon dioxide via the intermediates acetyl-CoA and acetyl-phosphate. Acetyl-CoA is formed by a pyruvate:ferredoxin oxidoreductase (41) and then is converted to acetate by the enzymes phosphate acetyltransferase and acetate kinase, with concomitant phosphorylation of ADP to ATP (42). In the case of lactate oxidation, no net formation of ATP is produced and thus a proton-driven ATP production is necessary to conserve energy (1). Energy conservation for growth of *D. vulgaris* in lactate and sulphate is proposed to occur through a mechanism known as “hydrogen cycling”. This mechanism considers that the electrons released by the oxidation of lactate to pyruvate and of pyruvate to acetyl-CoA are used by a cytoplasmic hydrogenase to produce hydrogen that, after diffusing through the membrane, is oxidised by the periplasmic hydrogenase to reduce sulphate. The proton release in the periplasm generates a proton gradient (vectorial electron transport) that can be used by ATP synthase to produce ATP (43). This model has been a matter of debate since the existence of cytoplasmic hydrogenases in *Desulfovibrio* sp. remains unknown and because hydrogen formation from

lactate oxidation is not an energetically favourable process (44). However, the analysis of the recently sequenced *D. vulgaris* Hildenborough genome gives support to the hydrogen cycling mechanism, since it contains genes encoding two orthologs of cytoplasmic-facing membrane-bound hydrogenase complexes (EchABCDEF and CooMKLXUHF). In *D. vulgaris*, these hydrogenases were proposed to be associated with production of H₂ from the oxidation of lactate or pyruvate (20).

In addition to hydrogen cycling, other mechanisms such as cycling of formate or CO may be operative for energy conservation. In *D. vulgaris*, three formate dehydrogenases oxidize formate to CO₂ and H⁺. Two of these enzymes are periplasmic and the other one is membrane associated (45, 46). The mechanism of formate cycling suggests that this compound is formed in the cytoplasm from lactate oxidation and then diffuses to the periplasm where is oxidized by the formate dehydrogenases. The protons are released in the cytoplasm and the electrons are donated to the periplasmic cytochromes *c* (20). The mechanism of CO cycling suggests that CO is formed from pyruvate (generated from lactate oxidation) by an unknown enzyme and is subsequently converted to CO₂ and H₂, by a cytoplasmic CO dehydrogenase and a CO-dependent hydrogenase, whose genes are present in the *D. vulgaris* Hildenborough genome. The H₂ produced may subsequently be oxidized by the periplasmic hydrogenase for sulphate reduction (47).

Electron carriers and membrane-bound electron transport complexes

In *Desulfovibrio* sp. the terminal reductases are cytoplasmic located and consequently not involved in membrane charge translocation. The reducing equivalents from electrons donors must therefore be transferred through electron carriers, and are used in the cytoplasmic sulphate reduction. *Desulfovibrio* sp. contain a high number of periplasmic and membrane-bound *c*-type cytochromes that mediate this electron transfer

(1). The periplasmic *c*-type cytochromes include the monohaem cytochrome c_{553} (proposed to accept electrons from iron-hydrogenases, formate dehydrogenases and lactate dehydrogenases) and multihæm cytochromes, such as the dimeric dihaem Split-Soret, the octahæm cytochrome c_3 and type I (TpI c_3) and type II tetrahaem cytochromes c_3 (TpII c_3). TpI c_3 was the first cytochrome isolated from a *Desulfovibrio* sp. and accept electrons from periplasmic hydrogenases (48, 49). The periplasmic cytochromes c_{553} and c_3 transfer electrons to membrane-bound complexes that contain a soluble cytochrome subunit of the c_3 family as electron acceptor. For example, Hmc is a transmembrane complex present in *D. vulgaris* Hildenborough that contains a 16-hæm cytochrome (HmcA) (49). The 9-hæm cytochrome *c* (9HcA), isolated from *D. desulfuricans* ATCC 27774, is also part of membrane-bound complex (9Hc) and is highly expressed on cells grown with sulphate (50). A third example is the Tmc complex in which the cytochrome *c* subunit is a TpII c_3 (TmcA) (51). The cytochrome *c* subunits of these three complexes can accept electrons from periplasmic hydrogenases via TpI c_3 (49). *Desulfovibrio* sp. contains other transmembrane complexes such as Qmo, which lacks the periplasmic cytochrome *c* subunit, and is proposed to transfer electrons from the menaquinone pool to APS reductase (52), and the DrsMKJOP complex, probably involved in the transfer of electrons to the dissimilatory sulphite reductase. Qmo and Drs complexes may be essential for sulphate reduction given their strong conservation among sulphate reducers (53).

Desulfovibrio contains also low molecular mass proteins such as ferredoxins, flavodoxins and rubredoxins which are involved in electron transfer processes. Ferredoxins are distinguished by the type of iron-sulfur cluster which they comprise. In *D. gigas*, ferredoxin I contains one [4Fe-4S] whereas ferredoxin II harbours one [3Fe-4S] cluster (54). Flavodoxins contain FMN as cofactor and rubredoxins have one [Fe-S(Cys)₄] centre (55, 56).

1.2.2 - *Desulfovibrio* in environment and bioremediation

SRB have been recognized for a long time to be associated with microbial corrosion that occurs in many industrial systems such as water supplies, drinking water distribution systems and petroleum and gas industry. Within the group of SRB, the majority of the strains present in the oil and gas reservoirs belong to the *Desulfovibrio* genus. In oil and gas industries the pressure within the reservoirs is maintained by injection of water or gas. The combination of the organic components that result from oil degradation (electron donors) with the sulphate (electron acceptor) present in water promotes the growth of SRB (native or introduced with the water injections), which are responsible for production of hydrogen sulphite in the oil and gas reservoirs, in a process named souring. Hydrogen sulphide decreases the quality of oil and gas and causes corrosion of the pipelines and processing equipment due to the reaction with Fe^{2+} to form a mixture of iron sulphides which are corrosive products (57). Strategies for control of souring in oil reservoirs have been developed and the most reliable is nitrate injection. The presence of nitrate stimulates nitrate-reducing, sulphide-oxidizing and nitrate-reducing bacteria that remove sulphide with the production of nitrite, which has an inhibitory effect on SRB (57). Nitrite is a strong competitive inhibitor of the dissimilatory sulphite reductase (DsrAB) of *Desulfovibrio*, since this enzyme binds tightly nitrite and slowly reduces it to ammonia (preventing the normal physiological function of DsrAB, *i. e.* reduction of sulphite to sulphide) (57, 58). *D. vulgaris* Hildenborough prevents this inhibitory event by the action of pentahaem nitrite reductase (NrfAH) but the efficiency of this detoxification system is dependent on the time required for the reduction of all nitrite content to ammonia, given that the protons and electrons from sulphate reduction are temporary diverted to the periplasmic nitrite reduction, which could lead to the collapse of the electrochemical gradient. A *nrfAH* mutant of *D. vulgaris* cannot survive in the presence of millimolar

concentrations of nitrite and thus SRB lacking this enzyme are even more sensitive to nitrite inhibition (57, 58).

Although *Desulfovibrios* are a problem for oil and gas industries, they constitute a group of microorganisms with great potential for bioremediation. The interactions between sulphate reducers and metals and metalloids has been of interest since these organisms have the capacity to reduce several toxic metals such as uranium(VI), copper(II), chromium(VI) and manganese(II). Also, the sulphides produced during SRB growth are able to precipitate several heavy metals into insoluble metal sulphides. The ability of these bacteria to reduce these toxic metals depends on the metal concentration in solution. The effect of heavy metals on sulphate reducers can be stimulatory at lower concentrations and inhibitory at higher concentrations. The reduction of these soluble metals into insoluble forms allows their removal from contaminated waters and waste streams which is performed in bioreactors (59). Hydrogenases and/or cytochromes are involved in the enzymatic reduction of the metals and metalloids by sulphate reducers. In *D. vulgaris* the tetrahaem cytochrome c_3 is involved in reduction of U(VI) to U(V) and both the cytochrome c_3 and the Fe-hydrogenase can reduce Cr(VI) to Cr(III) (60).

1.2.3 - *Desulfovibrio* in humans

Anaerobic fermentation in the intestine leads to the formation of products such as hydrogen, propionate, carbon dioxide, lactate and succinate, which can be utilized by *Desulfovibrio*. Sulphate is also available in the intestine derived from food, water and unabsorbed amino acids. Some intestinal bacteria also acquire sulphate from host glycoproteins, including mucins secreted by the gut epithelial cells (7). *Desulfovibrio* is the predominant genus of the sulphate reducing bacteria found in the microbial flora of the intestine of humans and animals. Transcriptional analysis

showed that *Desulfovibrios* are ubiquitous in human faeces but the number of these microorganisms varied with age (7).

Hydrogen sulphide produced by *Desulfovibrio* is toxic at high concentrations being a cause of DNA damage and inhibition of butyrate oxidation, an essential process for colon epithelial cells. The inhibition of the latter process is associated with ulcerative colitis, a form of inflammatory bowel disease (IBD), an acute and chronic illness of the small intestine and of the colon. Patients with ulcerative colitis and Crohn's disease (another form of IBD) seem to have a higher number of *Desulfovibrios* in their faeces than healthy individuals (61).

Some *Desulfovibrio* sp. were also isolated from abscesses of the brain, intra-abdominal and abdominal walls and pyogenic liver and from periodontal mouth lesions (7). Cases of bacteremia caused by *D. fairfieldensis* and *D. desulfuricans* were also reported (62), showing that *D. fairfieldensis*, is highly resistant to certain antimicrobial drugs (62, 63). *Desulfovibrio* strain FH26001/95, isolated from liver abscesses, was shown to grow with dissolved oxygen concentrations up to 50 μM . This tolerance to oxygen may be a strategy of *Desulfovibrio* to survive in the blood and tissues, where the concentration of dissolved oxygen is approximately 110 μM (64).

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Part I

Chapter 2

Desulfovibrio and oxygen



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2.1 – Sulphate reducing bacteria in oxic habitats

Sulphate reduction has long been recognized as an anoxic process performed by strict anaerobic sulphate reducing bacteria, including the *Desulfovibrio* species. However, measurements of bacterial sulphate reduction and dissolved oxygen in several microbial mats show that sulphate reduction occurs in oxygenated zones. Cyanobacterial mats are the habitat of many SRB species, and constitute a good example of the occurrence of extreme shifts regarding oxygen and sulfide concentrations (1, 2). In a cyanobacterial mat of a saline evaporation pond (Baja California, Mexico), the deeper zones are occupied by SRB of the genus *Desulfobacter* and *Desulfobacterium*, whereas *Desulfovibrio* and *Desulfococcus* are found in more oxic zones (upper zones) (3). In the case of a hypersaline cyanobacterial mat from Solar Lake (desert lake in the Sinai, Egypt), SRB are preferentially localized in a permanent oxic zone, being *Desulfonema* and *Desulfococcus* the predominant groups (4). The presence of these microorganisms in cyanobacterial mats indicate that they are able to deal with oxygen concentrations of approximately 1 mM (1, 2).

In biofilms, SRB of the genus *Desulfovibrio*, *Desulfomicrobium*, *Desulfonema*, *Desulforegula*, *Desulfobacterium* and *Desulfobulbus* were also found in the oxic/anoxic interface (5). Furthermore, *Desulfovibrio cuneatus* was isolated from the oxic uppermost layer of sediments of the oligotrophic Lake Stechlin (6) and *Desulfovibrio aerotolerans* was isolated from an activated sludge (sewage treatment), which had been oxygenated at 100 % of air saturation for 120 h (7).

2.2 - *Desulfovibrio* responses to oxygen

The presence of oxygen in *Desulfovibrio* habitats imposes a great challenge to these microorganisms because as anaerobes, they should not survive in these environments. However, their study show that they have systems for detoxification of reactive oxygen species (ROS) which cause

damage to a variety of biological targets. In fact, oxygen is able to freely cross the biological membranes and, inside the cell, it abstracts electrons from exposed redox centres of electron transfer proteins, leading to the formation of the several reactive oxygen species, such as superoxide ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (8). Hydrogen peroxide further reacts with free cellular Fe^{2+} , in the so called Fenton reaction, resulting in the formation of the hydroxyl radical (OH^{\cdot}), which is a powerful oxidant and damages DNA (8, 9). ROS also cause oxidation of protein cysteinyl amino acid residues, oxidation of solvent-exposed [4Fe-4S] clusters of enzymes, leading to iron release from the cluster and protein inactivation, and peroxidation of membrane lipids, eventually resulting in loss of membrane integrity (8).

To respond to oxygen, *Desulfovibrio* employ several strategies, *e.g.*, the formation of cell aggregates which occurs in both natural habitats and pure cultures (7, 10, 11). In response to oxygen gradients, some *Desulfovibrio* sp. migrate to the deeper anoxic zones of cyanobacterial mats. However, a different behavior is found for two strains, *D. oxyclinae* and *D. desulfuricans* CSN. Experiments performed in capillary tubes show that these bacteria migrate in the direction of air or oxygen bubbles, with the formation of ring-shaped bands around these bubbles. Furthermore, oxygen is completely consumed by these bacteria allowing the re-establishment of anoxic conditions (10, 12). *D. vulgaris* Hildenborough has a similar response, given that in a capillary tube with an oxygen gradient, the cells form a focused band near an oxygen concentration of 0.02-0.04 % and consume the oxygen in this zone (13). In all these strains, oxygen causes aerotactic band formation, which might be associated with a mechanism of oxygen sensing. In fact, *D. vulgaris* Hildenborough has transmembrane methyl-accepting protein (DcrA), containing a *c*-type haem in the periplasmic domain, which may function as an oxygen and/or redox potential sensor in *D. vulgaris* (14), and a protein containing a cytoplasmic

located hemerythrin-like domain (DcrH) is also proposed to function as an O₂ sensor (15). Furthermore, the presence in the *D. vulgaris* genome of genes encoding other members of the chemotaxis protein family suggests that these proteins could also be involved in oxygen sensing (16).

Although *Desulfovibrio* sp. are described to consume oxygen, no growth using oxygen as the only electron acceptor was, so far, described. Also, there are, until now, no reports of pure cultures of *Desulfovibrio* where sulphate is reduced in the presence of high oxygen concentrations. *D. desulfuricans* CSN reduces O₂ using H₂ as electron donor, in a process that is coupled to formation of ATP, but not to aerobic growth. The oxygen reduction rates measured in washed cells of *D. desulfuricans* CSN are significantly high, and can even be compared to those of some aerobic bacteria (17). Furthermore, oxygen is reduced prior to nitrate, when both are available as electron acceptors for *D. desulfuricans* CSN (18). Reduction of oxygen coupled to oxidation of other electron donors as for example lactate, pyruvate or thiosulphate, is also performed by *D. desulfuricans* CSN and other *Desulfovibrio* strains (e.g. *D. desulfuricans* ATCC 27774 and *D. desulfuricans* DSM642). Hence, the ability of reducing oxygen, using different types of electron donors, varies between the species (19).

2.3 - Proteins involved in the response to oxidative stress

Several genes coding for enzymes putatively involved in oxygen sensing, detoxification of ROS, reduction of oxygen to water and oxidative damage repair are predicted in the genomes of *Desulfovibrio* sp. Some of these enzymes were already shown to cope with ROS (Figure I-2.1) (20, 21). For example, the repair of 8-oxoguanine-DNA bases (a mutagenic oxidative damage product of guanine) in *D. vulgaris* Miyazaki F, is performed by MutM protein, a DNA glycosylase/lyase whose gene is induced by oxidative stress (23). The availability of free iron in the cell is maintained by ferritin

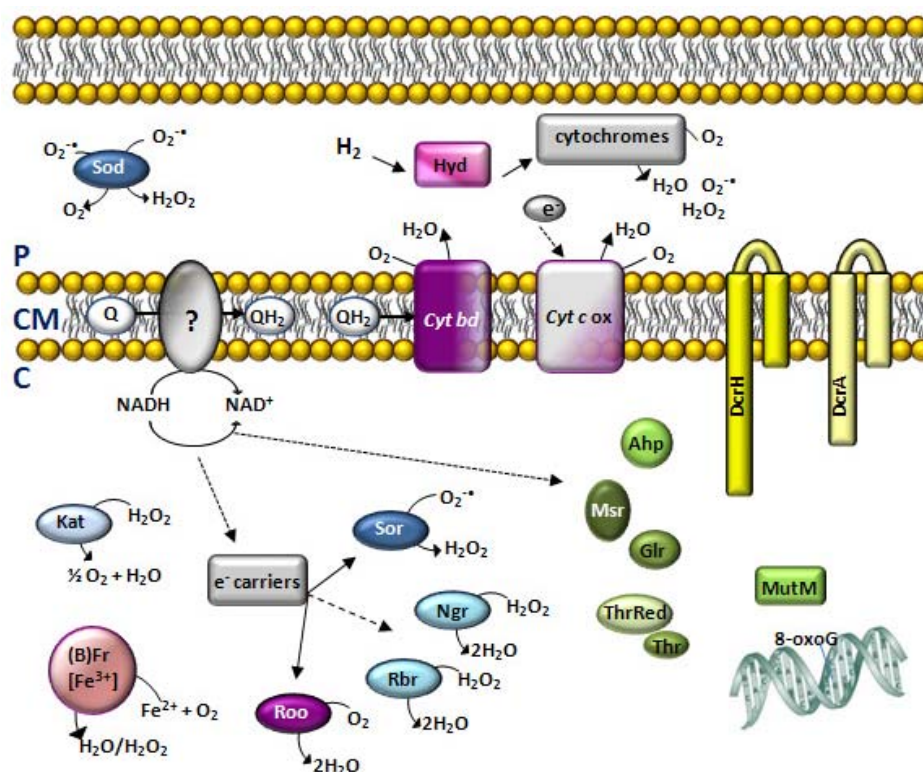


Figure I-2.1 - General model for oxygen defence in *Desulfovibrio* sp. (Adapted from (21)). Electrons for ROS generation can be generated by autooxidation of cytochromes or flavoproteins and by oxidation of lactate (cytoplasm), formate (periplasm) and chemical oxidation of sulphide. Blue proteins are involved in ROS detoxification (Sod: superoxide dismutase; Sor: superoxide reductase; Kat: catalase; Ngr: nigerythrin; Rbr: rubrerythrin); purple proteins are involved in oxygen reduction (Roo: rubredoxin:oxygen oxidoreductase; *Cyt bd*: *bd*-type quinol oxidase; *Cyt c ox*: haem-copper oxygen reductase) green proteins are involved in repair systems (Msr: methionine sulfoxide reductase; ThrRed/Thr: thioredoxin reductase/thioredoxin; Glr: glutaredoxin; MutM: DNA repair protein); yellow proteins are involved in oxygen sensing (DcrA and DcrH). (B)fr: (Bacterio)ferritin; Hyd: hydrogenases; e⁻: electron; Q/QH₂: oxidised/reduced quinone pool; P: periplasm; CM: cytoplasmic membrane; C: cytoplasm. DcrH topology adapted from (15) and DcrA topology adapted from (22).

and bacterioferritin enzymes that can reversibly store ferric iron as an oxyhydroxide mineral in their internal cavity. Oxygen is used to rapidly oxidize the ferrous iron in the catalytic (bacterio)ferritin di-iron site, and the ferric iron formed enters the protein cavity where is stored, therefore

avoiding the formation of ROS via the Fenton reaction. The bacterioferritin of *D. desulfuricans* ATCC 27774 is a 24 subunit enzyme with one iron-coproporphyrin III per dimer and one catalytic diiron site per monomer (24). Ortholog proteins involved in damage repair by disulfide reduction (thioredoxins/thioredoxin reductases, glutaredoxin), iron-sulphur cluster synthesis and reduction of methionine sulfoxides (methionine sulfoxide reductase) are encoded in the genomes of *D. vulgaris* Hildenborough and *D. desulfuricans* G20 (16).

2.3.1 - Detoxification of reactive oxygen species

Desulfovibrio sp. have several enzymes specialized in scavenging ROS, such as the superoxide dismutase (Sod) and catalase (Kat), present in all aerobic microorganisms (25). Sod catalyses the disproportionation of superoxide to hydrogen peroxide and catalase performs the disproportionation of hydrogen peroxide to water and oxygen (26). *D. gigas* has a catalase and a constitutive periplasmic iron-containing Sod (26), while *D. desulfuricans* ATCC 27774 contains a cyanide sensitive copper zinc containing-Sod and a catalase (27). Hydrogen peroxide is also detoxified by NAD(P)H dependent peroxidases such as rubrerythrin (Rbr) and nigerythrin (Ngr), that catalyse the two-electron reduction of hydrogen peroxide to water. Electrons from rubredoxin are donated to both peroxidases (28, 29). The hybrid-cluster protein (Hcp) of *D. desulfuricans* ATCC 27774 is also an enzyme with peroxidase activity that is involved in oxidative stress protection (30). In *D. vulgaris* Hildenborough and *D. desulfuricans* G20, additional protection against hydrogen peroxide is proposed to be provided by thiol-specific peroxidases, namely thiol peroxidase and bacterioferritin co-migratory protein (Bcp) and hydroperoxidases (AhpC and AhpF) (16).

The removal of superoxide in *Desulfovibrio* can also be performed by the superoxide reductase enzyme (Sor), which catalyses one electron

reduction of superoxide to hydrogen peroxide. Two cytoplasmic enzymes with Sor activity were identified in *Desulfovibrio*, the desulfoferredoxin (Dfx, also known as rubredoxin oxidoreductase (Rbo)) that contains two mononuclear non-haem iron centers (center I and II) (31) and neelaredoxin (Nlr), which contains one iron metal active site with homology to center II of Dfx (32). Interestingly, Nrl has Sod activity in addition to Sor activity (27, 32).

2.3.2 - Oxygen reduction systems

Cytoplasmic oxygen reduction chain

Desulfovibrio sp. such as *D. gigas* (33) and *D. salaxigens* Mast1 (34) are able to store high amounts of polyglucose and these carbon reserves are metabolized to produce ATP, via substrate level phosphorylation, leading to the production of NADH. In *D. gigas* this process is coupled to NADH re-oxidation by a cytoplasmic electron transfer chain that reduces oxygen to water (33, 34). This electron transfer chain consists in a NADH:rubredoxin oxidoreductase (Nro), a rubredoxin (Rd) and a rubredoxin:oxygen oxidoreductase (Roo). In this system, the small cytoplasmic electron carrier rubredoxin, acts as the bridge for electron transfer from Nro to Roo (35-37). Roo is a flavodiiron protein, functional as a homodimer, containing per monomer one FMN, two iron atoms and two unusually modified tetrapyrroles, uroporphyrin I and a derivative of mesohaem IX (35, 38). However, recent studies demonstrated that Roo-like enzymes also have nitric oxide reductase activity and are involved in resistance to nitrosative stress (39, 40).

Periplasmic oxygen reduction

D. vulgaris Marburg reduces oxygen to water in the periplasm, using hydrogen as electron donor, in a process that was proposed to involve electron transfer from hydrogenases to oxygen reducing cytochromes *c* (41).

This hypothesis was further supported with studies in *D. vulgaris* Hildenborough showing that both the Fe-hydrogenase and cytochromes are involved in periplasmic oxygen reduction (42).

Membrane-bound oxygen reductases

The sequencing of the cytochrome c_{553} of *D. vulgaris* Myasaki revealed that the gene encoding cytochrome c_{553} was located in the vicinity of a gene encoding for a haem-copper oxygen reductase, suggesting for the first time the presence of an aerobic respiratory-like haem-copper oxygen reductase in *Desulfovibrio* (43). Terminal membrane-bound oxygen reductases of the aerobic respiratory chains catalyse the reduction of oxygen to water. These enzymes are divided in three main groups: i) the haem-copper oxygen reductases (also named cytochrome *c* oxidases), which couple the reduction of oxygen to translocation of protons across the membrane (mitochondrial- or cytoplasmic-membrane, in the case of eukaryotes or prokaryotes, respectively) (44); ii) the *bd*-type oxygen reductases (quinol oxidases) and iii) the alternative oxidases. Contrary to the haem-copper oxygen reductases, the two latter enzymes do not translocate protons across the membrane (45, 46).

The prokaryotic members of the haem-copper oxygen reductase family have up to four subunits (I-IV). They all share a catalytic subunit (subunit I) with a copper ion (Cu_B) and two haem groups with distinct spin states. The high-spin haem and the copper ion form the catalytic center where oxygen reduction to water occurs, and the low-spin haem serves as the final electron donor to this center. Subunit II may contain a binuclear copper center (Cu_A) and, in several cases, an additional haem center is present in the C-terminal domain (44, 47). The electrons for oxygen reduction can be delivered from a variety of electron donors such as soluble cytochromes *c* and soluble high potential iron-sulphur proteins (HiPIPs) and membrane-bound quinols.

Haem-copper oxygen reductases are divided in three families, A (A1 and A2), B and C (Figure I-2.4), according to amino acid sequence conservation, catalytic center properties and amino acid residues that form the proton channels (48, 49). Type A and B have a conserved subunit II that may include or not the binuclear center. In the case of enzymes that receive electrons from soluble electron donors, the binuclear center serves as the primary electron acceptor and, in the case of enzymes in which electrons are transferred through membrane quinols, no binuclear site is present. Type A1 family includes the mitochondrial and mitochondrial-like enzymes, that contain two proton channels, D and K, in subunit I. The D channel is composed by eight conserved amino acids (AspI-124 (D), AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193 and GluI-278, *Paracoccus denitrificans* numbering) and the K channel, that leads directly to the binuclear site, is composed of four conserved residues (LysI-354 (K), ThrI-351, SerI-291, *Pa. denitrificans* numbering). In A2 type enzymes, these residues are conserved, with the exception of the glutamate present at the end of the D-channel, which is replaced by a tyrosine and a serine (48, 49). In enzymes of the B type family, the residues forming these channels are not conserved, but an alternative K-channel has been proposed (48, 49). Type-C enzymes (comprising *cbh3* oxygen reductases) have only some conserved residues of the alternative K-channel and comprise, besides subunit I, two distinct subunits with one and two haems *b*, respectively (48, 49).

The *bd*-type oxygen reductase is a quinol-oxygen oxidoreductase distinct from the haem-copper reductases. It contains three haem redox centers: haem *b*₅₅₈, located in subunit I and haem *d* and *b*₅₅₉, located in subunit II (45).

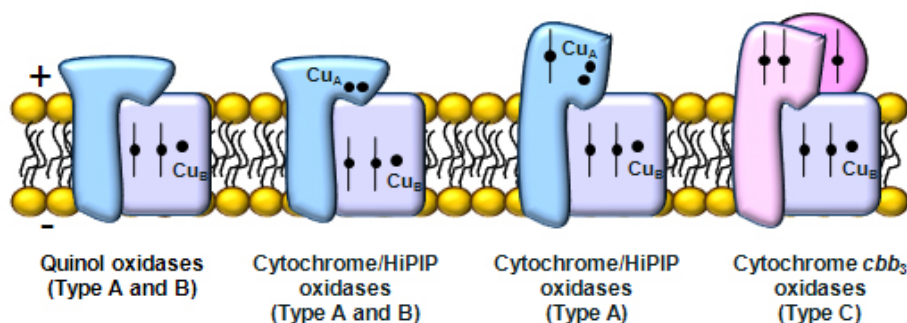


Figure I-2.4 – Schematic representation of the several types of the haem-copper oxygen reductases (Adapted from (49)).

The genomes of *D. vulgaris* Hildenborough, *D. vulgaris* PD4, *D. vulgaris* Miyasaki and *D. desulfuricans* G20 encode genes for haem-copper and *bd*-type oxygen reductases. However, in *D. desulfuricans* ATCC 27774, only the *bd*-type seems to be present. None of the proteins was, so far, isolated from these particular strains, but the characterization of the recombinant subunit II of the *D. vulgaris* Hildenborough haem-copper oxygen reductase was provided in the course of this work (Part II, chapter 2). *D. gigas* also contains a *bd*-type oxygen reductase, which was isolated from cells grown in a sulphate/fumarate medium, and shown to perform the complete reduction of oxygen to water (50). The *bd*-reductase, together with a quinol:fumarate oxidoreductase, isolated from *D. gigas*, form a minimal electron transfer chain that consume oxygen in the presence of duroquinone and succinate (51).

Although genes encoding oxygen reductases are present in *Desulfovibrio* sp., the physiological role (energy conservation and/or oxygen detoxification) of these enzymes in *Desulfovibrio* is still unknown.

2.4 - Transcriptomic and proteomic response to oxidative stress

With the availability of the genome sequences of *Desulfovibrio* sp. the global transcriptomic and proteomic analysis of these microorganisms started to come forth, providing a better understanding of the global cellular response under several stress conditions (52).

The proteomic analysis of the response of *D. vulgaris* Hildenborough to oxygen exposure (1 h of continuous bubbling with pure oxygen, corresponding to approximately 880 μ M of dissolved oxygen) shows that a decrease in the abundance of several proteins occurs, including proteins involved in nucleic acid synthesis, cell division and protein folding/stabilization. In accordance, when exposed to oxygen, *D. cuneatus* STL1 develops atypically elongated cells and cell division is inhibited (53, 54). Interestingly, iron containing proteins such as rubrerythins and superoxide reductase are down-expressed, and its repression was proposed to limit the availability of free metals thus avoiding the generation of ROS. An increase in the abundance of other proteins putatively involved in oxygen defence (Fe-hydrogenase, bacterioferritin co-migratory protein and glutaredoxin) and in iron-sulphur biosynthesis and/or repair was also observed (55). The repression of genes encoding proteins involved in energy metabolism was also observed in a transcriptional analysis, performed under similar oxidative stress conditions (56). The genes encoding thioredoxin, thioredoxin oxidoreductase, methionine sulfoxide reductases were up-regulated but, in contrast with the proteomic analysis, rubrerythrin and the superoxide reductase did not exhibit significant transcriptional alterations (56).

The oxidative stress response of *D. vulgaris* Hildenborough in two other conditions was also evaluated by microarray studies. In these experiments, cells were submitted to air (21 % oxygen, for 240 minutes) or to low oxygen concentrations (0.1 % oxygen for 240 minutes) (57). Exposure to 0.1 % oxygen results in a reduction of the cell growth rate but the cells

retain viability, while the presence of air causes drastic reduction of cell growth and viability. Several differences are observed between these two conditions. Gene products involved in central metabolism (such as sulphate reduction, ATP synthesis, pyruvate to acetate conversion) have no significant transcriptional alteration in cells exposed to 0.1 % oxygen, whereas exposure to air causes repression of these genes. Also, *sod* exhibits no significant expression change in cells exposed to 0.1 % oxygen while its transcription is largely increased in cells exposed to 21 % oxygen. Furthermore, in *D. vulgaris* cells submitted to 0.1 % oxygen, there is no modification in the transcription of the *roo* gene while genes belonging to the predicted peroxide regulon (PerR) (e.g. alkyl hydroperoxide reductase C, rubrerythrins, peroxide responsive regulator perR) are up-regulated. The genes encoding bacterioferritin, *tmc* transmembrane cytochrome *c₃* operon, *dviK* DNA-binding response regulator and cytochrome *cydAB* operon are also up-regulated and their expression correlates with PerR regulon expression, suggesting that these genes are under the control of the regulator PerR (57). In overall, these studies show that the response of *Desulfovibrio* to oxidative stress depends on oxygen concentration and the proteomic and transcriptomic data report that the majority of the genes, whose expression was modified, encode hypothetical proteins or proteins with unknown functions, thus showing that a significant number of oxygen protective enzymes remain to be identified.

2.5 - References

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Tetrapyrrole biosynthesis

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3.1 - Tetrapyrroles in life

Tetrapyrroles are the most abundant pigments in nature and are essential for life, being involved in a wide variety of biological processes (*e.g.* electron transfer processes, light harvesting, respiration and oxygen-binding). They consist of four pyrrole rings attached to one another, bridged by methine groups ($=CH-$) producing, in general, a macrocycle. In the case of corrinoids, one of the ring carbons is lost as a result of a ring contraction process (Figure I-3.1). The basic unsubstituted macrocycle suffers different modifications on all eight positions of the four pyrrole rings, to give several modified tetrapyrroles, which are subsequently chelated with a specific metal ion. The resulting metal-tetrapyrroles can be subdivided in various groups: haems (Fe), sirohaem (Fe), chlorophylls (Mg), bacteriochlorophylls (Mg), vitamin B₁₂ (Co), coenzyme F₄₃₀ (Ni) and haem *d*₁ (Fe). Linear tetrapyrroles (*e.g.* bilirubin, biliverdin), contrary to the latter macrocycles, do not contain metal ions and have only three bridged carbons (Figure I-3.2) (1, 2).

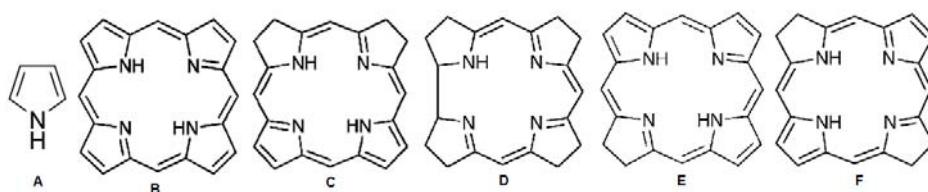


Figure I-3.1 - A - Pyrrole unit; Unsubstituted macrocyclic tetrapyrrole cores: B - Porphyrin (from the ancient greek word *porphura* that describes the colour purple), C - Isobacteriochlorin, D - Corrin, E - Chlorin, F - Bacteriochlorin (structures from (3)).

Historical perspective

It was almost 200 years ago that Pellitier and Caventou first used the word chlorophyll to describe the pigment that was responsible for the green

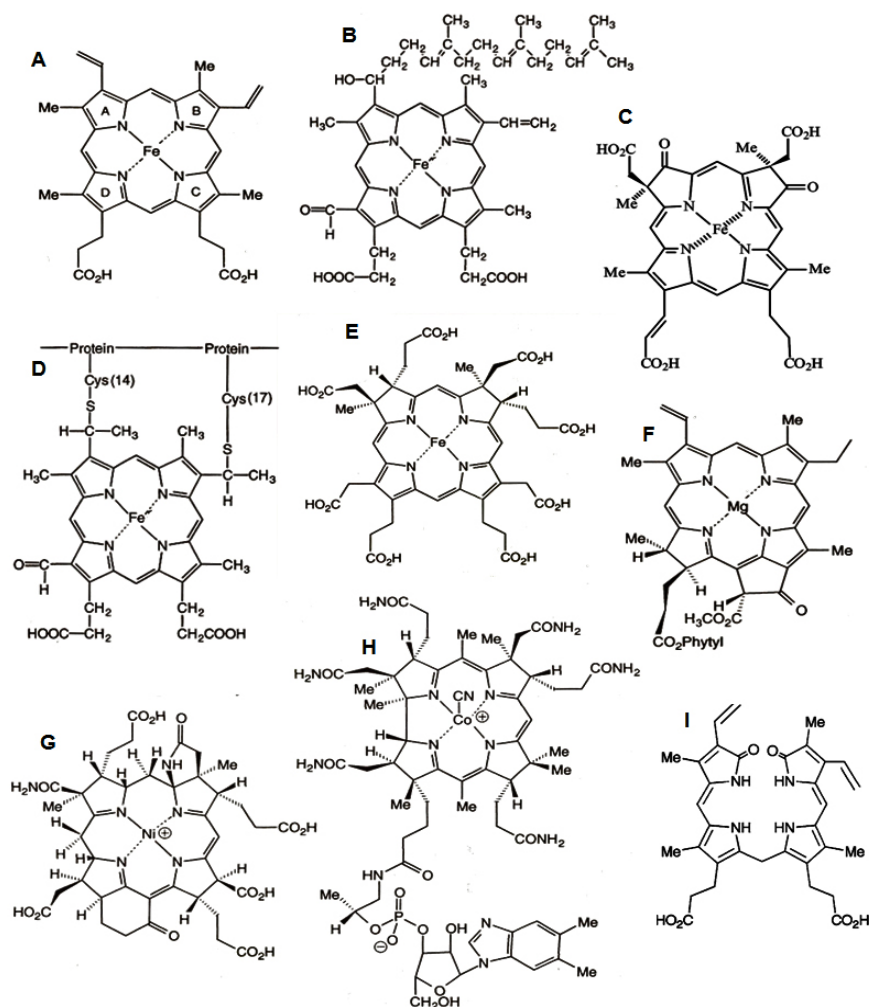


Figure I-3.2 - Examples of modified tetrapyrroles found in nature. A - Haem *b*, B - Haem *a*, C - Haem *d*₁, D - Haem *c*, E - Sirohaem, F - Chlorophyll *a*, G - Coenzyme F₄₃₀, H - Cyanocobalamin (vitamin B₁₂), I - Bilirubin (linear tetrapyrrole) (A, B, D-H from (1), C from (4) and I from (3)).

colour of leafs and approximately 40 years later, in 1864, Stokes made the observation that the chlorophyll fraction consisted of two different types, namely *a* and *b*. The finding that these molecules were organic complexes containing magnesium was made in 1907 by Willstätter (Noble Prize in Chemistry, 1915) and in 1925, Keilin found that haem was also an organic complex, but containing a different metal (iron), which was further confirmed by Fischer and Kämmerer. That these compounds could be a

system of porphyrins with four pyrrole rings linked together, forming a macrocycle, was originally proposed by Küster, in 1912 (3); however, it was only with the extensive work of Willstätter and Fischer that haem and chlorophyll structures were determined. The elucidation of the biosynthetic pathway of these “pigments of life” took several decades and resulted from the work of several groups (1).

In 1926, Minot and Murphy (both awarded with the Nobel Prize in Medicine, 1934) found that raw liver was an effective treatment for pernicious anaemia. This discovery led to the beginning of a quest to isolate the active agent responsible for this treatment. The work of Mary Shaw Shorb, together with Merck scientists, showed that the active agent of liver extracts could also stimulate growth of *Lactobacillus lactis*. This compound, isolated in 1948, in the form of red coloured crystals, was named vitamin B₁₂. Further studies showed that B₁₂ was produced by some microorganisms and, in 1955, Dorothy Hodgkin (Nobel Prize in Chemistry, 1964) was successful in unravelling the structure of B₁₂, a cobalt-containing molecule of the tetrapyrrole family that presents a high level of structural complexity (3).

Nomenclature of tetrapyrroles

The suggestion for a system of porphyrin nomenclature was initially given by Hans Fischer. It considers eight peripheral positions (1-8) and four *meso*-carbon positions (α , β , γ and δ) (Figure I-3.3). The different ways in which the several substituents can be arranged in the macrocycle of the porphyrin yields different “type-isomers”, which are represented in Roman numbers.

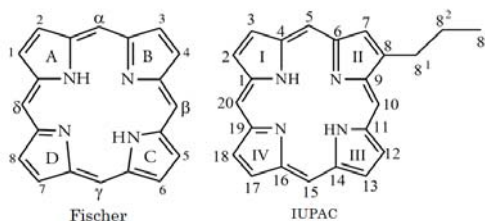


Figure I-3.3 - The two nomenclature systems of the macrocyclic tetrapyrroles (4).

For example, uroporphyrinogen has eight substituents, four acetic acid and four propionic acid groups and consequently there are four possible type isomers, I-IV. However, only type I and III occur in natural systems. A more systematic nomenclature was subsequently introduced, by IUPAC (International Union of Pure and Applied Chemistry), and brought consistency within corrin macrocycles, by numbering all the carbon atoms (Figure I-3.3) (1, 4).

3.2 - How are tetrapyrroles synthesised?

All tetrapyrroles originate from a common biosynthetic pathway which starts with the formation of the universal tetrapyrrole precursor, δ -aminolaevulinic acid (ALA), a linear five carbon molecule. Only three enzymatic steps are required to transform eight molecules of ALA into the tetrapyrrole macrocycle, uroporphyrinogen III. At the level of uroporphyrinogen III, the first of several bifurcations of the pathway occurs leading to the formation of precorrin-2, the branch point for sirohaem, haem d_1 , vitamin B₁₂ and cofactor F₄₃₀, and protoporphyrin IX. The latter is the precursor of haems and chlorophylls (Figure I-3.4) (2, 5).

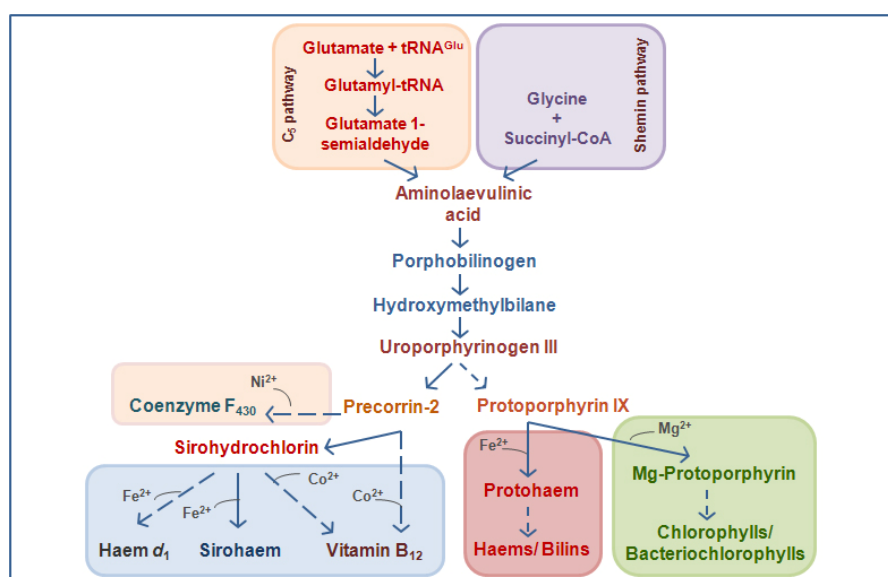


Figure I-3.4 - General tetrapyrrole biosynthetic pathway. Adapted from (6).

In eukaryotic cells, tetrapyrrole biosynthesis takes place in two different organelles. In animals and yeast, haem synthesis occurs partially in the mitochondria and partially in the cytosol whereas in plants, synthesis of both haem and chlorophyll occurs in plastids and mitochondria. In these organisms, haem biosynthesis involves both soluble and membrane-organelle associated proteins (2). In prokaryotes, the majority of the haem biosynthetic steps is performed by cytoplasmic enzymes, although the last steps are, in general, catalysed by membrane-associated enzymes. Furthermore, α -type haem biogenesis requires periplasmic proteins and integral membrane proteins with periplasmic catalytic domains (3).

3.2.1 - Overview on the early steps of the pathway

Aminolaevulinic acid and Porphobilinogen

Aminolaevulinic acid can be synthesized by two alternative routes: the Shemin pathway (also called C₄-pathway), in the case of animals, yeast, fungi and α -proteobacteria, and the C₅-pathway, that occurs in plants, algae and most prokaryotes (figure I-3.4) (7, 8). The photosynthetic *Euglena gracilis* is, so far, the only case reported in which both pathways are used to produce tetrapyrroles (9). In the Shemin pathway, ALA is formed by condensation of succinyl-CoA and glycine, in a one step reaction performed by a pyridoxal-5'-phosphate (vitamin B₆)-dependent enzyme, named ALA synthase and which is encoded by the gene *hemA*. The C₅-pathway starts with the C₅-skeleton of glutamate, which is bound to a tRNA^{Glu} by a glutamyl-tRNA synthetase. The resulting glutamyl-tRNA^{Glu} is reduced by a NADPH-dependent glutamyl-tRNA reductase (encoded by the gene *hemA* or *gltR*, to distinguish from the *hemA* of the Shemin pathway) to yield glutamate 1-semialdehyde. This compound is subsequently transaminated to produce ALA, in a pyridoxal-5'-phosphate-dependent reaction, catalysed by the glutamate 1-semialdehyde aminotransferase (encoded by *hemL*

gene) (see (2, 5, 6) for reviews). ALA is the substrate for porphobilinogen synthase (also named ALA dehydratase and encoded by the *hemB* gene) which catalyses the asymmetric condensation of two ALA molecules to produce the pyrrole derivate, porphobilinogen (PBG) (Figure I-3.5). This enzyme has two distinct binding sites for the substrate: the so called A-site, which recognizes the ALA molecule that will give rise to the acetic-acid side chain of PBG (A-side ALA), and the P-site, that binds the ALA molecule that will generate the propionic acid chain (P-side ALA) (10).

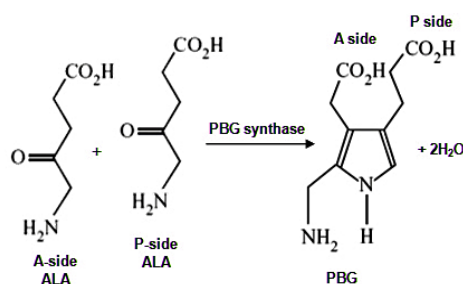


Figure I-3.5- Porphobilinogen synthase catalysed reaction.
Adapted from (11).

Porphobilinogen synthases are diverse in their requirement for zinc or magnesium in the active site, and for magnesium in the allosteric binding site. If present, allosteric magnesium seems to be involved in conformation changes in the active site, stabilization of the oligomeric structure of the protein and increase of the enzyme V_{\max} (12). Interestingly, the protein from *Rhodobacter capsulatus* does not use any metal ion for catalysis. In addition, *R. capsulatus* PBG synthase forms a stable and fully functional hexameric structure, while the majority of the PBG synthases form mainly homo-octameric structures (13, 14). Based on the available X-ray structures of the *Escherichia coli*, *Pseudomonas aeruginosa* and yeast PBG synthases, acquired with several inhibitors and substrate analogs, two alternatives ways for substrate binding were proposed (15-19). The initial event is common to both, consisting in the binding of P-side ALA to the P-site of the enzyme, via a Schiff-base with a highly conserved lysine residue (Lys247,

E. coli numbering). The subsequent step is the binding of the second A-side ALA. In zinc-dependent enzymes, A-side ALA binds to the metal centre and in the case of non-metal dependence, A-side ALA binds to the A-site via Schiff-base with another conserved lysine residue (Lys195, *E. coli* numbering) (10).

Hydroxymethylbilane

The polymerization of four PBG molecules, to form the linear tetrapyrrole 1-hydroxymethylbilane (HMB), is performed by the enzyme PBG deaminase (encoded by *hemC* gene). The *E. coli* PBG deaminase is a monomeric protein containing a dipyrromethane cofactor (derived from two PBG molecules) covalently attached to Cys242. This cofactor acts as a primer for the sequential addition of four PBG molecules, through enzyme-substrate intermediate complexes called ES, ES₂, ES₃ and ES₄ (Figure I-3.6) (20-22). The assembly of the dipyrromethane cofactor occurs in the first catalytic cycle of PBG synthase. The precursor of this cofactor is HMB, the product of the reaction itself. The apoenzyme binds first to HMB via a thioether linkage to Cys242 to form the ES₂ intermediate complex (Figure I-3.6), which is then able to complete the catalytic cycle, through the sequential addition of two PBG molecules to form ES₄. Once the six molecules of PBG are assembled, HMB is hydrolyzed and the dipyrrole remains covalently bound to the enzyme, maintaining the protein in the active holo form (23). HMB is very unstable and auto-chemically cyclizes to form uroporphyrinogen I isomer. This intermediate is not considered to be physiologically useful; however, the *D. gigas* rubredoxin:oxygen oxidoreductase, an enzyme involved in oxygen reduction (see also Part I, Chapter 2), contains as cofactor uroporphyrin I, the oxidized form of uroporphyrinogen I (24).

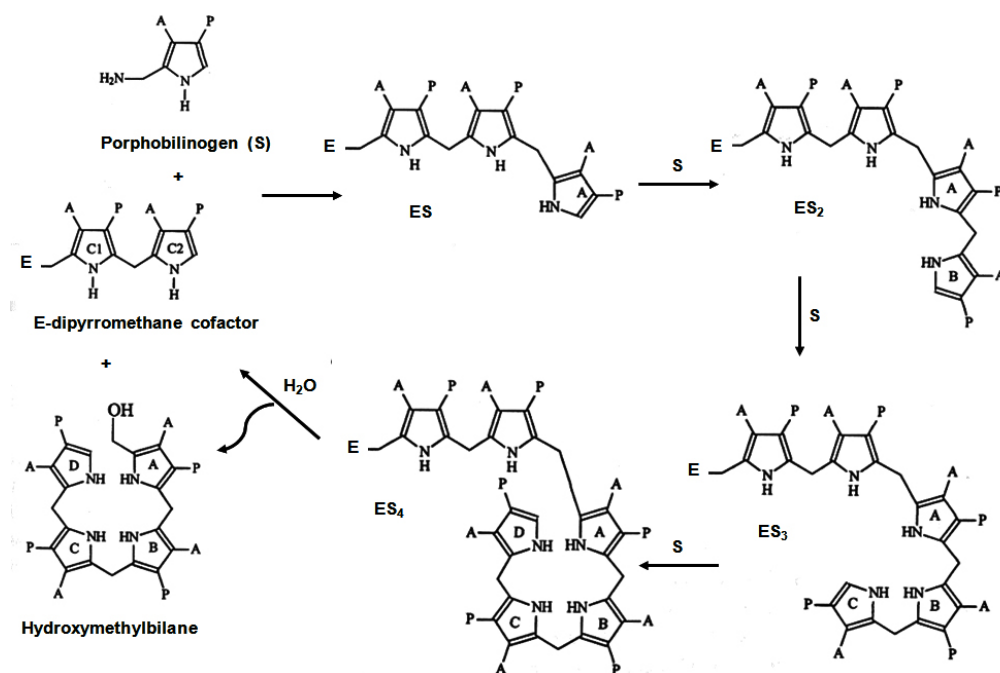


Figure I-3.6 – Catalytic cycle of porphobilinogen deaminase. The enzyme (E) reacts with the porphobilinogen substrate (S) that is deaminated, to form the complex ES. Three S molecules are successively added to the poly-pyrrole growing chain, forming ES₂, ES₃ and ES₄. The product hydroxymethylbilane is cleaved and the enzyme is regenerated. A- Acetate ($-\text{CH}_2\text{COOH}$); P - Propionate ($-\text{CH}_2\text{CH}_2\text{COOH}$) (Adapted from (23)).

Uroporphyrinogen III

In the presence of uroporphyrinogen III synthase (encoded by gene *hemD*), HMB is cyclized with concomitant inversion of the fourth ring (ring D) of the porphyrin to give the asymmetric uroporphyrinogen III. Mechanistic studies proposed that the rearrangement of ring D involves a spiro-intermediate (with a bond between carbons 16 and 20) (Figure I-3.7) (see (6, 11) for reviews). The uroporphyrinogen synthases of *Human*, *E. coli*, *Thermus thermophilus* and *Bacillus subtilis* are all monomeric proteins. The structures of the *Human* and *T. thermophilus* enzymes show that they are comprised of two domains with similar folds, connected by a flexible short linker segment, where the active side lies (25-28). In the parasite *Plasmodium falciparum* and in the bacterium *Leptospira interrogans*, PBG deaminase is a bifunctional enzyme having also uroporphyrinogen III

synthase activity. However, the reaction mechanisms of these enzymes remains unknown (29, 30).

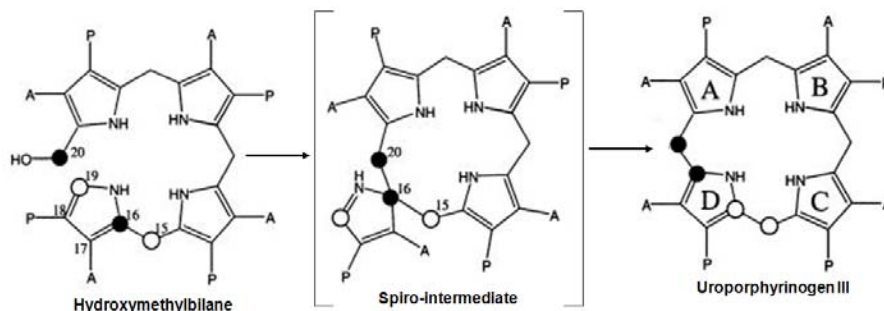


Figure I-3.7 – Proposed mechanism for the rearrangement of uroporphyrinogen III, catalysed by uroporphyrinogen III synthase. A - Acetate ($-\text{CH}_2\text{COOH}$); P - Propionate ($-\text{CH}_2\text{CH}_2\text{COOH}$). Adapted from (27).

3.2.2 - Overview on the late steps of the pathway

Uroporphyrinogen III is the last common intermediate of the pathway of all organisms that biosynthesise tetrapyrroles. From this point, the pathway branches to the formation of several tetrapyrrole derivatives. While decarboxylation of this macrocycle leads to formation of haems and chlorophylls, C-methylation leads to the synthesis of sirohaem, haem *d*₁, coenzyme F₄₃₀ and vitamin B₁₂. This overview will focus mainly in the sirohaem branch, with a brief review of the vitamin B₁₂ and haem branches.

Sirohaem and vitamin B₁₂

Sirohaem is the prosthetic group of sulphite and sirohaem-containing nitrite reductases. The synthesis of sirohaem from uroporphyrinogen III involves three different reaction steps: i) methylation of uroporphyrinogen III to generate precorrin-2; ii) dehydrogenation of precorrin-2 to form sirohydrochlorin and iii) chelation of iron into sirohydrochlorin to give sirohaem (Figure I-3.8). These steps are achieved by the action of different enzymes, depending on the organisms. In *B. megaterium*, for example, sirohaem is synthesized by three independent enzymes, encoded in the

sirABC operon: the S-adenosyl-methionine:uroporphyrinogen III methyltransferase (SUMT) SirA, the NAD⁺-dependent precorrin-2 dehydrogenase SirC and the sirohydrochlorin ferrochelataze SirB (31). In the case of *E. coli* and *Salmonella thyphimurium*, the conversion of uroporphyrinogen III to sirohaem is catalysed by one single multifunctional enzyme, the sirohaem synthase (CysG) (32, 33). In the yeast *Saccharomyces cerevisiae*, two enzymes are required: a protein with SUMT activity, named Met1p, and the bifunctional dehydrogenase/ferrochelataze, Met8p (34).

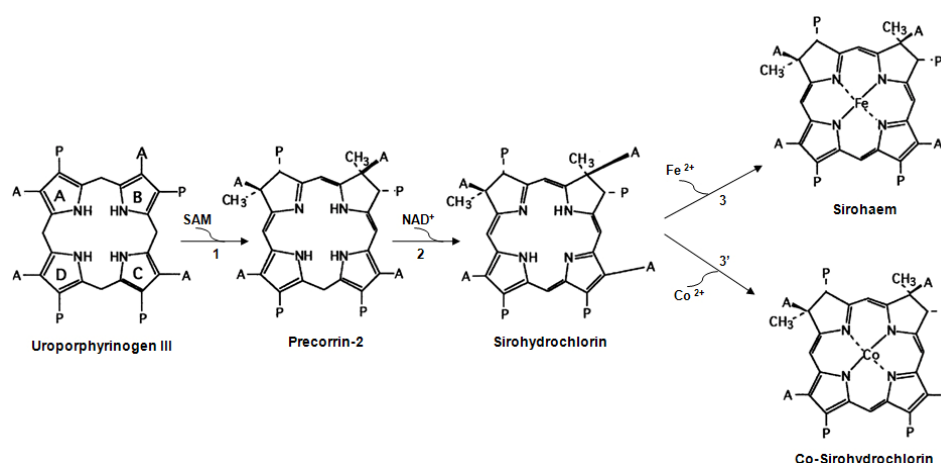


Figure I-3.8 - Sirohaem and Co-sirohydrochlorin synthesis from uroporphyrinogen III. 1 - S-adenosyl-methionine:uroporphyrinogen III methyltransferase, 2 - Precorrin-2 dehydrogenase; 3 - Sirohydrochlorin ferrochelataze; 3' - Sirohydrochlorin cobaltochelataze. A - Acetate (—CH₂COOH); P - Propionate (—CH₂CH₂COOH). Adapted from (35).

The biosynthesis of vitamin B₁₂ (cobalamin) requires almost thirty different enzymes and the *de novo* synthesis of this extremely complex molecule is restricted to certain prokaryotic microorganisms (*e.g.* species of the genera *Bacillus*, *Clostridium*, *Propionibacterium*, *Pseudomonas* and *Salmonella*). Vitamin B₁₂ is largely found in nature in the forms of 5'-deoxyadenosylcobalamin (coenzyme B₁₂) and methylcobalamin. The commercially available form of vitamin B₁₂ is cyanocobalamin, in which the adenosyl or methyl groups are replaced by a cyano group, as the result of the extraction process from the bacterial cultures. In prokaryotes, vitamin

B₁₂-dependent enzymes include methionine synthase, ribonucleotide reductase and proteins involved in anaerobic fermentation of 1,2-propanediol, ethanolamine and glycerol. Although animals and humans do not synthesize cobalamin, they have two enzymes that require vitamin B₁₂ as co-factor, namely the methylmalonyl-CoA mutase and the methionine synthase. Plants, on the other hand, seem to neither synthesize nor use vitamin B₁₂ (36-38).

In prokaryotes there are two different pathways for the biosynthesis of the corrin ring of vitamin B₁₂, the aerobic (oxygen-dependent) and the anaerobic routes. The first step which is the methylation of uroporphyrinogen III to generate precorrin-2 by SUMT, is common to both routes and to sirohaem synthesis. In the anaerobic pathway (Figure I-3.8), precorrin-2 is further dehydrogenated by a NAD⁺-dependent precorrin-2 dehydrogenase to form sirohydrochlorin, which is subsequently chelated with cobalt, by a cobaltochelataase, to yield cobalt-sirohydrochlorin. Further modification of the latter compound leads to the formation of vitamin B₁₂. In the aerobic route, the insertion of cobalt occurs at later stage, after several modifications of precorrin-2 at the level of hydrogenobyrrinic acid *a,c*-diamide (36).

Precorrin-2

SUMT is the enzyme responsible for two successive S-adenosyl-methionine (SAM) dependent methylations of carbons 2 (C2) and 7 (C7) of uroporphyrinogen III, to form precorrin-2. The initial methylation is at C2 position, leading to the formation of the intermediate precorrin-1, which is subsequently methylated at position C7. The *Ps. denitrificans* SUMT enzyme was the first isolated and is a single functional homodimeric protein, encoded by gene *cobA* (therefore SUMT is frequently referred to as CobA). In some bacteria such as *Clostridium josui* (39), *Selenomonas ruminantium* (40) and *Lactobacillus reuteri* (41), the *cobA* gene is fused with the *hemD* gene. Both the *B. megaterium* and the *Ps. denitrificans*

SUMTs display substrate inhibition at concentrations above 0.5 and 2 μM , respectively, which is proposed to be a regulatory mechanism of vitamin B₁₂ synthesis (42, 43). The crystal structure of *Ps. denitrificans* SUMT shows that the protein monomer has two different domains connected by a single linker. S-adenosyl-methionine/homocysteine (SAM/SAH) binds in a large pocket located between the N and C-terminal domains of each subunit of the dimer and uroporphyrinogen III is proposed to bind in a channel in the N-terminal domain, which is surrounded by several flexible loops (44). The apo-form (without SAM) of *Thermus thermophilus* SUMT exhibits a similar overall structure; however the displacement of a flexible loop near the active site suggests that the protein was crystallized in a “closed” form that prevents the access to the substrate, and it was proposed that this conformation may reflect a way of controlling the enzyme activity (45).

Sirohydrochlorin

Precorrin-2 is oxidized to sirohydrochlorin by a NAD⁺-dependent precorrin-2 dehydrogenase. In the multifunctional sirohaem synthase CysG, precorrin-2 dehydrogenase activity is linked to the N-terminal domain (CysG^B). The sirohydrochlorin ferrochelatase activity is also associated to the CysG^B domain while the C-terminal domain (CysG^A) is linked to SUMT activity (32, 33). The structure of *S. typhimurium* CysG revealed that CysG^A and CysG^B form two distinct modules, with two and three domains, respectively. S-adenosyl-homocysteine is bound in the active site cleft of CysG^A at the junction of the two domains, and NAD⁺ binds to the CysG^B domain I (33).

The bifunctional precorrin-2 dehydrogenase/ferrochelatase, Met8p, of yeast is a homodimer with two NAD⁺-binding domains, an interwinded central domain and two helical C-terminal domains. This protein has a single active site and the nearby located aspartate residue number 141 plays an essential role in both activities (46).

The only available structure of a single functional precorrin-2 dehydrogenase is from the *B. megaterium* SirC (47), which is a homodimeric protein that shows structure similarities to both CysG and Met8p NAD⁺-binding domains. The monomer is constituted by three domains and the active site harbors the NAD⁺-binding site, formed by several strictly conserved residues that are predicted to interact with precorrin-2. Given the essential role of Asp141 for metal chelation in Met8p, site directed mutagenesis studies of SirC, where the equivalent serine residue was replaced by an aspartate, were performed. However, this mutation failed to generate an enzyme with chelatase activity (47).

Sirohaem and cobalt-sirohydrochlorin

The metal insertion in the tetrapyrrole ring, catalysed by chelatases, involves distortion of the tetrapyrrole macrocycle, proton release from two pyrrole nitrogens and formation of the metal-ligand bond (48). The chelatase family of enzymes is divided in three classes according to size and energy requirement: Class I chelatases which are ATP-dependent enzymes composed of three subunits (*e.g.* the protoporphyrin IX:magnesium chelatase BhlH-I-D of the chlorophyll synthesis and the hydrogenobyrinic acid *a,c*-diamide cobaltochelatase of aerobic vitamin B₁₂ synthesis); Class II chelatases which are monomeric or homodimeric enzymes that do not require ATP for function (*e.g.* sirohydrochlorin cobaltochelatases CbiX^S, CbiX^L and CbiK, the sirohydrochlorin ferrochelatase SirB and the protoporphyrin IX ferrochelatase HemH); and Class III chelatases that are homodimeric multifunctional enzymes that do not use ATP (*e. g.* Met8p and CysG) (49).

S. thyphimurium and *E. coli* multifunctional CysGs are able to insert both iron and cobalt into sirohydrochlorin, although the latter organism does not produce vitamin B₁₂ *de novo* (50, 51). In *S. thyphimurium*, CysG is necessary for sirohaem and anaerobic vitamin B₁₂ synthesis but in the

absence of this protein, sirohydrochlorin can be chelated with cobalt or iron by CbiK, a class II anaerobic cobaltochelataase. Nevertheless, it was suggested that CbiK has a major role in vitamin B₁₂ rather than in sirohaem biosynthesis, given its preference for cobalt (50, 52).

The ability of inserting both iron and cobalt metal ions into sirohydrochlorin is also a feature of other anaerobic sirohydrochlorin cobaltochelataases (49). These comprise, besides CbiK (constituted of ~300 amino acids), the CbiX^L protein (~320 amino acids) and its archeal orthologue, CbiX^S (110-145 amino acids). The N- and C-terminal domains of CbiX^L have high amino acid sequence similarity with each other and with the CbiX^S. In contrast, low amino acid sequence similarity is found between the two CbiX (CbiX^L and CbiX^S) and CbiK. Interestingly, a [4Fe-4S] cluster is present in the CbiX^L enzymes of *B. megaterium* and *Synechocystis* (53). Although iron-sulphur clusters ([2Fe-2S]) have also been described in prokaryotic and eukaryotic protoporphyrin IX ferrochelataases (HemH) (54) and in the *Arabidopsis thaliana* SirB (55), the function of these clusters is still unknown.

To date, structural X-ray information of sirohaem-synthesizing ferrochelataases is only available for the multifunctional enzymes CysG and Met8p and the latter presents a different fold with no resemblance to the available structures of cobalt and ferrochelataases (46). Despite the low amino acid sequence similarity, circular dichroism spectra analysis of SirB, CbiK, CbiX and HemH, suggests that these enzymes may have a similar overall topology (49). The structure of *S. typhimurium* CbiK revealed that the protein adopts a bilobal topology with two domains, similar to the protoporphyrin IX ferrochelataase HemH (56), and that the active site contains two histidine residues (His145 and His207) considered to be important for proton removal and cobalt binding (57). Furthermore,

structural studies (58) with *S. typhimurium* CbiK (57) and *B. subtilis* HemH (56) show that the individual domains of the bilobal chelatases have topological architecture similar to the single-domain chelatases CbiX^S of *Archaeoglobus fulgidus* (59) and *Methanosarcina barkeri* (58). Previous studies suggest that the two domains of the bilobal enzymes originate from a common ancestral protein by gene duplication event and that the CbiX^S could represent a primordial chelatase. Also, the fact that the two catalytic residues are present in only one of the two domains of CbiK, HemH and CbiX^L suggest that only one of the duplicated domains retained the activity (49).

Haem

The haem branch of the tetrapyrrole biosynthetic pathway starts with the sequential decarboxylation of the four acetate side chains of uroporphyrinogen III (positions C2, 7, 12 and 18) to methyl groups, forming coproporphyrinogen III. This reaction is catalysed by uroporphyrinogen decarboxylase (encoded by gene *hemE*) (Figure I-3.9A) (6, 60). The second step is catalysed by the coproporphyrinogen oxidase enzyme that performs the decarboxylation of the two propionate side chains of the rings A and B of coproporphyrinogen to

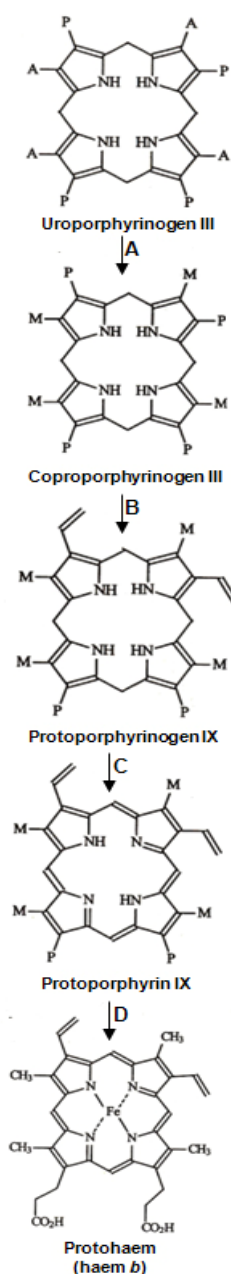


Figure I-3.9- Synthesis of haem *b* from uroporphyrinogen III.

A: $-\text{CH}_2\text{COOH}$; V: $-\text{CH}=\text{CH}_2$

P: $-\text{CH}_2\text{CH}_2\text{COOH}$; M: $-\text{CH}_3$.

Adapted from (6).

vinyl groups, generating protoporphyrinogen IX (Figure I-3.9B). Coproporphyrinogen oxidases differ on their oxygen requirement for catalysis and therefore are divided in two different types: oxygen-dependent (HemF) and oxygen-independent (HemN or HemZ) (6, 60). In general, the oxygen-independent HemN is a monomeric, [4Fe-4S] cluster-containing protein that belongs to the radical-SAM protein family (61).

The next step in the pathway is the oxidation of protoporphyrinogen IX to protoporphyrin IX by the enzyme protoporphyrinogen IX oxidase (Figure I-3.9C). Like coproporphyrinogen oxidase, two types of protoporphyrinogen IX oxidases have been identified: a flavin-containing oxygen-dependent enzyme (encoded by gene *hemY*) and a oxygen-independent enzyme (encoded by gene *hemG*) (6, 60). These enzymes can exist as soluble proteins, as in *B. subtilis* (62), or are membrane-bound like in eukaryotes (located in the inner mitochondrial membrane) and in some prokaryotes, where they are anchored to the cytoplasmic membrane. Protoporphyrin IX is subsequently chelated with ferrous iron to form protohaem IX (haem *b*) by the protoporphyrin IX ferrochelatase HemH (Figure I-3.9D) (48). *In vitro*, ferrochelatases are also able to use other divalent metals such as Co^{2+} , Zn^{2+} and Co^{2+} . The *Human* enzyme contains an [2Fe-2S]-cluster and is associated to the mitochondrial-membrane. Membrane-association and the presence of an iron-sulphur cluster is also observed in some prokaryotic ferrochelatases (48). Nevertheless, many ferrochelatases do not contain iron-sulphur clusters, such as the case of the *B. subtilis* ferrochelatase, which is a soluble protein and does not contain any prosthetic groups (56).

Haem *b* is the precursor of the other haem-types, namely of haems *a*, *c*, *o* and *d* (63). Haem *o* is synthesised by haem *o* synthase, a membrane bound enzyme that transfers the farnesyl group of farnesyl diphosphate to the 2-vinyl group of ferrous haem *b*. Haem *a* is synthesised from haem *o*, by a haem *a* synthase which is also a membrane protein (63). Haem *c*, the cofactor of *c*-type cytochromes, is found covalently attached to the protein

through two thioether bonds formed between two cysteine residues of a typical CxxCH motif in the protein, and the two vinyl groups of the haem (Figure 3.2D). c -Type cytochromes require specialised biogenesis systems for their formation due to the covalent attachment of the haem c to the apocytochrome, which occurs in the periplasm. A transmembrane transport is therefore necessary since both the haem and the apocytochrome are generated in the cytoplasm (64). In some Gram-negative bacteria such as *E. coli*, the Ccm system for cytochrome c maturation consists of eight proteins associated with the cytoplasmic membrane that are encoded by the *ccmABCDEFGH* operon (5).

3.3 - Tetrapyrroles of *Desulfovibrio*

The presence in a single organism of several multihaem c -type cytochromes, containing 4, 9 or even 16 c -type haems, is a feature of *Desulfovibrio* species. The tetrahaem cytochrome c_3 is the most abundant periplasmic cytochrome in these bacteria and is the electron acceptor for the periplasmic hydrogenases (see Part I, Chapter 1). The study of the cytochrome c_3 of *D. vulgaris*, isolated from cultures grown in the presence of labeled methionine, revealed that the methyl groups of the C2 and C7 of the haem c of cytochrome c_3 were originated from methionine (65) rather than ALA, as generally expected. However, the structure of the c -type haems of *Desulfovibrio* cytochromes c_3 is identical to other haems c thus suggesting the existence of an alternative biosynthetic pathway in *Desulfovibrio*. Subsequent studies by Ishida *et al.* (66) and Mathews *et al.* (67) confirmed the existence of a different tetrapyrrole pathway in these organisms, in which haem is made via precorrin-2. More recently, precorrin-2 was also shown to be an intermediate for haem biosynthesis in the methanogen *Methanosarcina barkeri* (68).

Proteins containing unusual tetrapyrrole cofactors are also found in *Desulfovibrio*. Uroporphyrin I is present in the rubredoxin:oxygen

oxidoreductase of *D. gigas* (24), and the bacterioferritin of *D. desulfuricans* ATCC 27774 contains iron-coproporphyrin III (69). Sirohaem is also present as cofactor of the dissimilatory sulphite reductase. In *D. vulgaris*, the latter tetrapyrrole exists in both the iron-chelated form, coupled to a [4Fe-4S] cluster, as in other sulphite reductases, and in the iron-free form, *i.e.* sirohydrochlorin, which is closely located to a [4Fe-4S] cluster, but not coupled to it (70). *Desulfovibrio* sp. produce cobalt-isobacteriochlorins containing proteins, whose oxidized form appears to be Co³⁺-sirohydrochlorin or a derivative of this macrocycle (71). Corrinoids such as guanylcobamide and hypoxanthylcobamide were also reported to be generated by *D. vulgaris*, and the addition of 5,6-dimethylbenzimidazole to the culture of *D. vulgaris* leads to the production of cyanocobalamin (72). Furthermore, *D. desulfuricans* LS was found to methylate mercury via methylcobalamin (73).

The presence of these unusual cofactors in *Desulfovibrio*, which are metal-chelated or metal-free intermediates of the tetrapyrrole pathway are, so far, only found in these bacteria. The work of Chapter 3, 4 and 5 of Part II of this thesis aimed at contributing to clarify how *Desulfovibrio* species synthesize these modified and apparently unique tetrapyrroles.

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Part II

Chapter 1

The anaerobe *Desulfovibrio desulfuricans* ATCC 27774 grows at nearly atmospheric oxygen levels[‡]

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Summary

Sulphate reducing bacteria of the *Desulfovibrio* genus are considered anaerobes, in spite of the fact that they are frequently isolated close to oxic habitats. However, until now, growth in the presence of high concentrations of oxygen was not reported for members of this genus. This work shows for the first time that the sulphate reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774 is able to grow in the presence of nearly atmospheric oxygen levels. In addition, the activity and expression profile of several key enzymes was analyzed under different oxygen concentrations.

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1.1 - Introduction

The bacterium *Desulfovibrio desulfuricans* ATCC 27774 belongs to the group of the sulphate reducing bacteria that derive their energy from anaerobic respiration. Although named after a single electron acceptor, *Desulfovibrio* species are able to use a wide variety of inorganic compounds, as electron acceptors (1). In particular, *D. desulfuricans* ATCC 27774 can also use nitrate as the terminal electron acceptor for anaerobic growth, performing a dissimilatory nitrate reduction that yields ammonia (2). It is well documented that sulphate reducing bacteria are usually found close to oxic habitats and several studies demonstrated that *Desulfovibrio* species survive prolonged exposure to oxygen, by the action of enzymes that enable them to cope with oxygen (3-5). Analysis of the *D. vulgaris* Hildenborough and *D. desulfuricans* G20 genome sequences shows that they contain genes for membrane bound oxygen reductases of the haem-copper and cytochrome *bd* types. Two oxygen-reducing enzymes have already been isolated from *D. gigas*: the membrane-bound terminal oxygen reductase of the cytochrome *bd* family (6) and a soluble oxygen reductase (ROO) that catalyses the transfer of electrons from rubredoxin to oxygen (7). Hence, *Desulfovibrio* species seem to have the enzymes required to grow under oxygen respiring conditions. Therefore, we undertook the study of the growth behaviour of a sulphate reducing bacterium in the presence of oxygen, with a *Desulfovibrio* species capable of growth in nitrate, *D. desulfuricans* ATCC 27774, thereby eliminating the possible chemical reactions of reduced sulphur compounds with oxygen which could, at least partially, mask the observations.

1.2 - Materials and Methods

Cell growth and cell fraction preparation. *D. desulfuricans* ATCC 27774 was grown anaerobically in a 3 L fermentor (Applikon, Biocontroler 4DI 1030), equipped with pH and pO₂ controllers, in lactate/nitrate medium (8) at

37 °C and under nitrogen (150 ml min⁻¹). Cell exposure to different percentages of oxygen (v/v) was achieved by replacing the nitrogen flux with the required amount of air by means of an Applikon gas mixer. The calibration of the O₂ electrode was performed at the same conditions used for growth. Cells were harvested from stationary phase by centrifugation (10000 x *g*, 20 min), resuspended in 10 mM MOPS buffer, pH 7.6, disrupted in a French Press and centrifuged for 30 min at 12000 x *g* to remove cell debris. The supernatant was centrifuged for 2.5 hours at 160000 x *g*, allowing the separation of the membrane and soluble fractions. The protein content of the fractions was determined using the BCA Protein Assay Kit (Pierce).

Haem analysis and enzymatic activities. UV–Visible absorption spectra of the membrane and soluble fractions prepared from cells of *D. desulfuricans* ATCC 27774 grown either anaerobically or in the presence of 18 % of oxygen were recorded in a Shimadzu UV-1603 spectrophotometer, at room temperature. Haem extraction was performed as described in (9) and analyzed in a Beckman HPLC system Module 167 with a Waters Nova-Pack C18 column. Type *aa3* cytochrome *c*:oxygen reductase from *Paracoccus denitrificans* and bovine myoglobin (Sigma) were used as standards for haems *a* and *b*, respectively.

Nitrite and nitrate reductase activities were performed anaerobically according to (8) and (10), respectively, using 1 mM benzyl viologen as electron donor. Peroxidase activity was measured spectrophotometrically in 10 mM Tris/HCl buffer, pH 7.4, using 31 µg of the soluble fraction, 1.2 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 33.5 mM H₂O₂, with an $\epsilon_{\text{DAB},460} = 1.68 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, which was experimentally determined in this work. Spectrophometric assay of SOD activity was done according to (11). Catalase activity was determined polarographically in a Clark-type oxygen electrode, YSI Model 5300, Yellow Springs, in 50 mM potassium phosphate buffer, pH 7, using 31 µg of soluble fraction and 335 mM H₂O₂ (12). The

oxidation of 5 mM of ascorbate mediated by 1 mM of tetra-methyl-p-phenylenediamine (TMPD) and its inhibition by 20 μ M of KCN, was measured in 10 mM MOPS buffer, pH 7.6 at 25 °C in the oxygen electrode. NADH:ferricyanide oxidoreductase and succinate:DCPIP oxidoreductase activities were followed spectrophotometrically, in 10 mM MOPS buffer, pH 7.6 at 30 °C. The reduction of 1 mM of $K_3[Fe(CN)_6]$ by 1 mM of NADH was monitored at 420 nm. For succinate:2,6-dichlorophenolindophenol (DCPIP) oxidoreductase activity, PMS (phenazine methosulphate)-coupled reaction of DCPIP was followed at 578 nm, by mixing 0.1 mM PMS, 0.150 mM DCPIP and 20 mM of freshly prepared succinate. Hydrogenase (13) and superoxide dismutase (SOD) (14) activities were assessed on Native Polyacrylamide Gel Electrophoresis (PAGE), using 100 μ g and 200 μ g of soluble fraction for hydrogenase and SOD detection, respectively.

1.3 - Results

Growth of *D. desulfuricans* ATCC 27774 in the presence of oxygen. In order to observe the ability of *D. desulfuricans* ATCC 27774 to grow in the presence of oxygen, the cells were cultured in lactate/nitrate medium under nitrogen, in a fermentor equipped with an oxygen electrode. It must be stressed that the medium did not include sulphate or any other sulphur containing compounds, thus eliminating chemical reactions of these compounds with oxygen. At $OD_{600} \sim 0.7$, cells were submitted for seven hours to different percentages of oxygen, namely 5, 15, 18 and 21 %, by replacing part of the nitrogen flux with the required amount of air. It was observed that cells of *D. desulfuricans* ATCC 27774 could grow at percentages of oxygen as high as 18 %, showing the growth curves behaviours similar to the control curve obtained under strictly anaerobic conditions (Figure II-1.1A). Upon supply of 21 % O_2 , cells stopped growing but did not exhibit cell death. Cells growing under oxygen atmosphere were

effectively consuming oxygen, with a rate that decreased once the stationary phase was reached (Figure II-1.1B). For all oxygen conditions tested, cells harvested from the stationary phase grew when re-inoculated in fresh media and cultured under strict anoxic conditions. This result shows that the bacterium did not lose the capacity for anaerobic growth, contrary to what was observed for other *Desulfovibrio* strains (15).

Microscope analyses revealed no contaminants in unstained and safranine or methylene blue stained preparations of *D. desulfuricans* ATCC 27774 cells exposed to air. In addition, all *D. desulfuricans* ATCC 27774 cells exposed to air revealed motility and cell division behaviour similar to cells cultured under anoxia. The cells presented the typical morphology of *Desulfovibrio* characterized by quite small cells with straight form, which is quite distinct

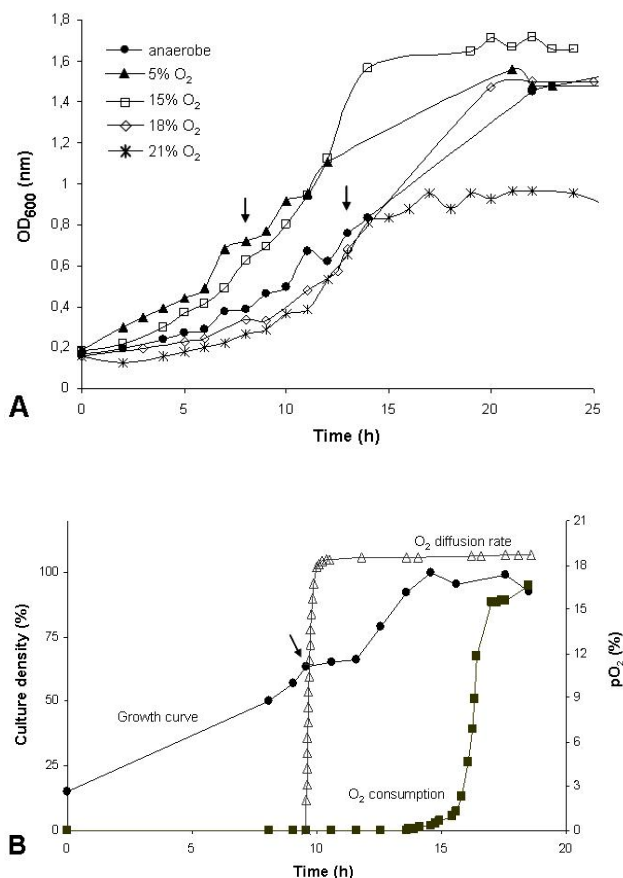


Figure II-1.1 - *D. desulfuricans* ATCC 27774 grows in aerated cultures exhibiting oxygen consumption. **Panel A:** Growth curves of *D. desulfuricans* ATCC 27774 obtained under anaerobiosis and with different concentrations of oxygen. At OD₆₀₀~0.7 (black arrow), cells were exposed to an atmosphere containing 5, 15, 18 and 21 % O₂, for approximately 7 h. **Panel B:** Oxygen consumption of *D. desulfuricans* ATCC 27774 cells grown in the presence of 18 % oxygen. Culture density values were normalized considering the maximum absorbance measured at the stationary phase.

from most aerobes. Furthermore, the oxygen exposed *D. desulfuricans* ATCC 27774 cells did not develop an atypically elongated form neither tended to form aggregates, as observed for other *Desulfovibrio* strains when submitted to oxygen (15).

Analysis of oxygen detoxifying enzymes of *D. desulfuricans* ATCC 27774. Since *D. desulfuricans* ATCC 27774 performs nitrate respiration, the influence of oxygen on the activity of the nitrate and nitrite respiring enzymes was analyzed. The results showed that the presence of oxygen did not alter significantly the nitrate and nitrite reductase activities of *D. desulfuricans* ATCC 27774 (Table II-1.1).

Table II-1.1 - Comparison of several enzymatic activities measured in *D. desulfuricans* cells grown anaerobically (0 %) or in the presence of O₂ (18 %).

Activity	O ₂ (%)	
	0	18
Nitrate reductase ^a (nmol min ⁻¹ mg ⁻¹)	8	5
Nitrite reductase ^b (μmol min ⁻¹ mg ⁻¹)	46	49
SOD ^{a,c} (U mg ⁻¹)	49	73
Catalase ^a (μmol min ⁻¹ mg ⁻¹)	78	142
Peroxidase ^a (μmol min ⁻¹ mg ⁻¹)	3	3
NADH:ferricyanide oxidoreductase ^b (μmol min ⁻¹ mg ⁻¹)	8	7
Succinate:DCPIP oxidoreductase ^b (nmol min ⁻¹ mg ⁻¹)	59	61
TMPD: oxygen oxidoreductase ^b (nmol min ⁻¹ mg ⁻¹)	7	9
Inhibition of O ₂ consumption (%)	40	55

^a Soluble fractions; ^b Membrane fractions; ^cFor SOD, one unit of activity (U) is defined as the amount of enzyme required for inhibition of 50 % of cytochrome *c* reduction. Values are the average of three experiments.

To determine whether the haem content changed with the degree of oxygen exposure, UV-Visible absorption spectra of the oxidized and dithionite reduced membrane and soluble fractions prepared from *D. desulfuricans* ATCC 27774 cells grown with zero and 18 % oxygen were recorded. The spectra of the membranes displayed an absorption in the Soret region (422 nm) and peaks at 522 and 553 nm, indicating the presence of

haem *c*. Furthermore, only a slight change in the haem composition, reflected in the shift of the Soret band, was observed upon increase of oxygen concentration in both membrane and soluble fractions (data not shown). The HPLC haem analysis of the membrane fractions of the anaerobic and 18 % oxygen grown cells revealed the presence of non-covalently bound haem *b* but not haem *a*, thus suggesting that the amount of haem *a* is below detection or, as previously proposed for *D. gigas* (6), these organisms may contain a different type of haem *a*.

Three hydrogenase-activity stained bands, corresponding to the [Fe]-, [NiFe]- and [NiFeSe]-hydrogenases (16), were detected in the soluble fractions of *D. desulfuricans* ATCC 27774 cells grown either under anaerobic conditions or in presence of 18 % O₂ and their intensity did not vary upon exposure to oxygen (Figure II-1.2A). These results are in agreement with data obtained in *D. vulgaris*, which also showed that the expression of [NiFe]-hydrogenase and [NiFeSe]-hydrogenase is not up-regulated by oxygen (17). However, the increase in expression of the [Fe]-hydrogenase that occurs in *D. vulgaris* upon oxidative stress (17) could not be observed in *D. desulfuricans* ATCC 27774 cells upon exposure to oxygen.

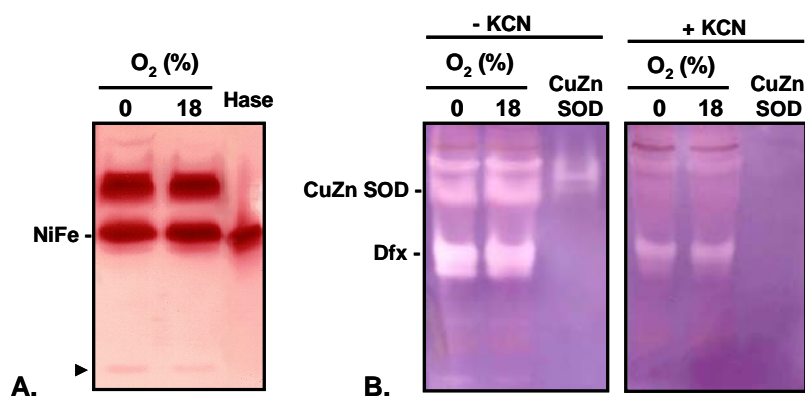


Figure II-1.2 - Native PAGE of the soluble fractions of *Desulfovibrio desulfuricans* ATCC 27774 grown anaerobically and under 18 % O₂, showing hydrogenase (A) and SOD (B) staining activities. The *D. desulfuricans* ATCC 27774 NiFe-hydrogenase and bovine CuZn-SOD (Sigma) were used as positive controls.

The presence of a wide range of enzymes involved in cell protection against oxidative stress in *Desulfovibrio* species is well documented (6, 7, 14, 15). In this study, catalase and SOD activities were enhanced by oxygen exposure while the peroxidase activity was not affected (Table II-1.1). The SOD gel activity revealed the presence of three distinct bands and their intensities with or without oxygen were similar (Figure II-1.2B). At present, the origin of the top band is unknown. The middle band is assigned to the putative Cu,Zn-SOD of *D. desulfuricans* ATCC 27774, which was inhibited upon addition of cyanide, and the lower band corresponds to desulfoferrodoxin (Dfx), a superoxide reductase, based on gel migration of the purified enzyme (14). These results are in accordance with those obtained for *D. gigas* and *D. vulgaris* that also show no significant change in SOD activity when the strains are exposed to oxygen (18). The expression of ROO, an enzyme proposed to be involved in O₂ detoxification (7, 19), was assayed by immunoblotting detection, and did not change upon exposure to 18 % oxygen (data not shown). Similarly, no major variations were observed in the protein level of *D. desulfuricans* ATCC 27774 bacterioferritin and its putative partner, rubredoxin II (data not shown), in spite of the fact that iron-storage enzymes of the ferritin family have been proposed to play a role in oxidative stress response.

The activities of membrane bound respiratory enzymes such as NADH:ferricyanide oxidoreductase and succinate:DCPIP oxidoreductase were also determined. The exposure of cells to oxygen did not increase the activity of these enzymes and oxygen consumption by membrane extracts, assessed through the reduction of oxygen with ascorbate-reduced TMPD, did not vary significantly under oxygenated conditions (Table II-1.1). The available genomes of several *Desulfovibrio* species reveal the presence of genes coding for haem-copper or *bd* type oxygen reductases. Although their presence in *D. desulfuricans* ATCC 27774 has not been yet demonstrated, it is probable that the genome encodes similar enzymes, thus explaining the

existence of the TMPD:oxygen oxidoreductase activity in the membrane extracts. The fact that only ~50 % of this activity was inhibited with KCN, a specific inhibitor of the haem-copper containing oxidases, suggests the coexistence in *D. desulfuricans* ATCC 27774 of haem-copper enzymes with other oxygen reductases, namely the *bd*-type enzymes, which are KCN insensitive.

1.4 - Discussion and conclusion

In this work, the oxygen reducing systems that enable *D. desulfuricans* ATCC 27774 to cope with oxygen, were characterized. Concerning SOD, the change in the activity of the enzyme followed a pattern similar to that observed for other *Desulfovibrio* strains also exposed to oxygen (18). In contrast with other *D. desulfuricans* strains, namely *D. desulfuricans* (strain Essex 6) and *D. desulfuricans* B-1388 (15) which are catalase negative, *D. desulfuricans* ATCC 27774 exhibits a positive catalase activity that increases upon exposure to oxygen. We also verified that in *D. desulfuricans* ATCC 27774 the expression of [Fe]-hydrogenase, desulfoferrodoxin, ROO, and bacterioferritin was not elevated by the increase of the oxygen concentration. Furthermore, the increase in the content of the periplasmic cytochromes *c* upon oxygen exposure detected in *D. vulgaris* (17) was not observed in *D. desulfuricans* ATCC 27774. Since the levels of oxygen utilized in this work were much higher than those used in other studies, it is possible that once a certain oxygen concentration is reached, the role of the protection systems changes accounting for the differences observed. Concerning the lack of increase in the activity of oxygen reductases of *D. desulfuricans* ATCC 27774 upon exposure to oxygen, it may result from a differential expression of these enzymes in response to oxygen levels (*e.g.* *bd* or *cbb₃* enzymes are preferentially expressed under quasi anaerobic conditions, while the other types of haem copper oxygen reductases have a higher expression under higher oxygen concentrations (20)). Studies on *D. gigas* also show that the

gene expression level of cytochrome *bd* is not significantly altered upon exposure to oxygen (21).

It has been shown that several sulphate reducing bacteria are able to survive exposure to oxygen, although within limited oxygen concentrations (4). For example, *D. desulfuricans* NCIB8301 can only grow at low oxygen pressures, with a drastically decrease of the cell yield at oxygen partial pressures greater than 0.4 % O₂ (22). For oxygen concentrations between 0.5 to 2 % O₂, weak growth is only observed for two strains of *D. desulfuricans* (Essex and CSN) and for *Desulfobacterium autotrophicum* DSM, which nevertheless show a strong decrease in cell viability and motility for oxygen concentrations higher than 2 % (23). A coculture of *D. oxyclinae* with the aerobe *Marinobacter sp.* consumed oxygen efficiently up to a supply of 5 % O₂. However, the portion of *D. oxyclinae* in the coculture decreased significantly upon aeration (24). Hence, although oxygen tolerance of various sulphate reducing bacteria is well established, the present work shows that *D. desulfuricans* ATCC 27774 exhibits the highest tolerance of all species so far examined, and constitutes the first example of a sulphate reducing bacterium with the ability to grow in a pure culture under nearly atmospheric oxygen levels.

1.5 - Acknowledgments

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The haem-copper oxygen reductase of *Desulfovibrio vulgaris* contains a dihaem cytochrome *c* in subunit II[‡]

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Summary

The genome of the sulphate reducing bacterium *Desulfovibrio vulgaris* Hildenborough, still considered a strict anaerobe, encodes two oxygen reductases of the *bd* and haem-copper types. The haem-copper oxygen reductase deduced amino acid sequence reveals that it is a Type A2 enzyme, which in its subunit II contains two *c*-type haem binding motifs. We have characterized the cytochrome *c* domain of subunit II and confirmed the binding of two haem groups, both with Met-His iron coordination. Hence, this enzyme constitutes the first example of a *ccaa3* haem-copper oxygen reductase. The expression of *D. vulgaris* haem-copper oxygen reductase was found to be independent of the electron donor and acceptor source and is not altered by stress factors such as oxygen exposure, nitrite, nitrate, and iron; therefore the haem-copper oxygen reductase seems to be constitutive. The KCN sensitive oxygen reduction by *D. vulgaris* membranes demonstrated in this work indicates the presence of an active haem-copper oxygen reductase. *D. vulgaris* membranes perform oxygen reduction when accepting electrons from the monohaem cytochrome *c*₅₅₃, thus revealing the first possible electron donor to the terminal oxygen reductase of *D. vulgaris*. The physiological implication of the presence of the oxygen reductase in this organism is discussed.

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*Footnote - It should be noted that *D. desulfuricans* G20 is in fact not related
to the *D. desulfuricans* strains, such as Essex and ATCC 27774.

2.1 - Introduction

Sulphate reducing bacteria of the *Desulfovibrio* genus are able to use a large diversity of inorganic compounds as electron acceptors (1). Although considered for many years as strict anaerobes, several *Desulfovibrio* strains show high tolerance to oxygen (2-4). This is particularly important, since sulphate reducers live in habitats close to the oxic/anoxic zones (5-7). In particular, *D. desulfuricans* ATCC 27774 was recently reported to be able to grow in the presence of nearly atmospheric oxygen levels (4). Several enzymes involved in the detoxification of reactive oxygen species (ROS) are proposed to sustain aerobic tolerance and to be involved in the reduction of oxygen to water (8). For example, the cytoplasmic rubredoxin:oxygen oxidoreductase (ROO), one of the first examples of the family of flavodiiron enzymes (9, 10), and the membrane-bound terminal oxygen reductase of the cytochrome *bd* family of *D. gigas* were shown to reduce oxygen to water (9, 11). Also, these organisms contain a plethora of systems that enable them to cope with reactive oxygen species, including superoxide dismutases and reductases, and catalases (8). However, there is still no clear evidence in *Desulfovibrio* species for an oxygen reduction coupled to oxidative phosphorylation. Genes encoding a haem-copper oxygen reductase and a *bd*-type oxygen reductase are present in the genomes of *D. vulgaris* Hildenborough and *D. desulfuricans* G20* as well as a gene encoding a protohaem IX farnesyltransferase. The latter protein synthesizes haem *o* from the protohaem IX (haem *b*) which is further modified to yield haem *a*, the cofactor present in several haem-copper oxygen reductases.

Oxygen reductases are the last complexes in the membrane-bound respiratory chains of aerobic organisms, and can be divided into three major groups: the haem-copper oxygen reductases (also generally called cytochrome *c* oxidases), the *bd*-type oxygen reductases, which are solely quinol oxidases, and the alternative oxidases which contain a di-iron catalytic site and occur in plants, fungi, protists and in some bacteria (12-16). The haem-copper

oxygen reductases catalyse the four electron reduction of dioxygen to water and use the free energy released from the oxidation of periplasmatic metalloproteins by dioxygen to pump protons across the membrane (15-19). Both the chemical reaction and the pumping of protons lead to the generation of a transmembrane difference of electrochemical potential, a key process in biological energy conversion.

The haem-copper oxygen reductases derive their name from the presence in the catalytic subunit (subunit I, the only subunit common to all members of this superfamily) of a centre constituted by a high-spin haem and a copper ion, where the reduction of molecular oxygen to water occurs; this subunit also contains a low-spin haem, the ultimate electron donor to the catalytic centre. The microbial enzymes have up to four subunits, whereas the eukaryotic enzymes are constituted up to thirteen subunits. The superfamily of haem-copper enzymes was divided into three families, A, B and C (19), according to amino acid sequence comparisons and to the conservation of amino acid residues proposed to be involved in the uptake and pumping of protons. This classification was later corroborated by the compilation and comparison of data acquired for the catalytic centre (20). The A family, was further subdivided into subfamilies A1 and A2. The A1 subfamily comprises the mitochondrial and mitochondrial-like microbial enzymes that have several conserved amino acid residues (in the so-called D and K channels) which were shown by various studies to be important for proton uptake and pumping. The enzymes of the A2 subfamily contain all those residues, with the exception of a glutamyl present at the end of the D-channel, in helix VI: instead, they have a tyrosyl and a seryl in the same helix, one turn below, which were proposed to play a role in proton conduction (19). Subunit II, conserved in A and B type enzymes, has either a binuclear average valence copper centre if it receives electrons from periplasm facing metalloproteins or no prosthetic group in the case of quinol

oxidases. In some cases, this subunit contains an extra C-terminal domain, which harbours a *c*-type haem (19).

In this article we show that the *D. vulgaris* haem-copper oxygen reductase is a member of the A2 type enzymes, that it is expressed under a variety of conditions, and demonstrate that subunit II contains two *c*-type haems. Furthermore, the lack of information on the nature of the electron donors to the *Desulfovibrio* haem-copper oxygen reductases led us to explore the possibility that, in *D. vulgaris*, the monohaem cytochrome c_{553} performs this role since this protein is encoded by the gene *cyf* located in the vicinity of the genes encoding the haem-copper oxygen reductase.

2.2 - Materials and Methods

Cloning and expression of the cytochrome *c* domain of the *D. vulgaris* Hildenborough haem-copper oxygen reductase. In *D. vulgaris* Hildenborough the haem-copper oxygen reductase subunit II is encoded by the gene DVU1812 (21). A truncated form of this gene was constructed to allow production of a protein comprising only the C-terminal region that contains binding motifs for two haems *c*. The correspondent DNA fragment, with 794 bp, was amplified by means of a PCR reaction using genomic DNA of *D. vulgaris* and the following oligonucleotides: 5'- CAA ACA TGC GCA TAT GCT TTC TGT C - 3' and 5'- GAG ACT CCT GAA AGC TTC ATG AC - 3' with restriction sites for NdeI and HindIII, respectively. The gene was cloned in pET-28a(+) (Novagen) to allow the insertion of a sequence that encodes a 6x-His tag. The gene was then subcloned in pET22b(+) (Novagen), previously digested with NcoI and HindIII, that further allows the expression of a PelB leader sequence. The truncated subunit II of the haem-copper oxygen reductase from *D. vulgaris*, from now on referred as cytochrome *c* domain, was then produced in *E. coli* BL21-Gold (DE3) (Stratagene) harbouring a plasmid with auxiliary genes for haem *c* production (pEC86-

ccmABCDEFGH) (22). The cells were grown, at 37 °C, in LB medium containing 50 µg/ml of kanamycin and 20 µg/ml of chloramphenicol, until an OD₆₀₀ ~0.7. At this point, 200 µM of IPTG (isopropyl-β-D-thiogalactopyranoside) and 50 µM of FeSO₄ were added to the medium. The culture was grown, at 37 °C, for extra 4 h after which the cells were collected by centrifugation.

Cloning and expression of the recombinant *D. vulgaris* Hildenborough cytochrome *c*₅₅₃. In the *D. vulgaris* Hildenborough genome, the cytochrome *c*₅₅₃ is encoded by the *cyf* gene (DVU1817) (21), that was amplified in a PCR reaction performed with genomic DNA of *D. vulgaris* and two oligonucleotides specifically designed for the *cyf* gene: 5' - GAG AAT TCC ATG AAA CGA GTT C -3', with a EcoRI restriction site, and 5' - TCG CTC GAG CTT GCT CAT GTA GTC - 3', with a XhoI restriction site. The gene was then cloned directly in pET-22b(+), sequenced to confirm the absence of errors and introduced in *E. coli* BL21-Gold (DE3) cells that also contained plasmid pEC86-*ccmABCDEFGH*. To produce the recombinant protein, cells were grown in LB medium supplemented with 50 µg/ml of kanamycin and 20 µg/ml of chloramphenicol, at 37 °C, until an OD₆₀₀ ~0.5. The expression procedure was done as above described for the production of the cytochrome *c* domain.

Protein purification. Cells overexpressing the cytochrome *c* domain or the cytochrome *c*₅₅₃ were resuspended in 20 mM TrisHCl buffer, pH 7.5 (buffer A) with 20 µg/ml of DNase and disrupted in a French Press. A high speed centrifugation, at 160000 x *g* for 1h, allowed the separation of the soluble fraction from the membranes, which was then loaded into a Chelating Sepharose High Performance column (GE Healthcare), previously equilibrated with NiCl₂ and with buffer A plus 400 mM NaCl. A linear gradient up to 400 mM imidazole was then applied and the cytochrome *c*

domain protein was eluted at ~250 mM imidazole, while the cytochrome c_{553} was eluted at ~280 mM imidazole. At this stage, the cytochrome c_{553} was found to be pure, as judged by SDS-PAGE (23). The fraction containing the cytochrome *c* domain protein was further purified in a Q-Sepharose High-Performance column (GE Healthcare) to which a linear gradient up to 1M NaCl in buffer A was applied, occurring the elution of a fraction with pure protein at ~250 mM NaCl.

Protein biochemical characterization. Protein concentration was determined by the bicinchoninic acid method (24) using protein standards from Sigma, and the hemochromopyridine method was performed according to the procedure described by Berry *et al.* (25). Protein molecular mass was assessed by gel filtration in a Superdex 200 column (GE Healthcare), according to the instructions of the manufacturer and using the commercially available standards of GE Healthcare.

UV-visible spectra were recorded, at room temperature, in a Shimadzu UV-1700 spectrophotometer. A redox mixture, containing 50 mM TrisHCl pH 7.5, 2.8 μ M of purified protein and 12 μ M of mediators, was titrated under argon atmosphere and continuous agitation. The redox mediators used were the following: N,N-dimethyl-p-phenylene-diamine ($E'_{0,7}$ = 340 mV), p-benzoquinone ($E'_{0,7}$ = 240 mV), 1,2-naphtoquinone-4-sulfonic acid ($E'_{0,7}$ = 215 mV), 1,2-naphtoquinone ($E'_{0,7}$ = 180 mV), trimethylhydroquinone ($E'_{0,7}$ = 115 mV), phenazine methosulphate ($E'_{0,7}$ = 80 mV), 1,4-naptoquinone ($E'_{0,7}$ = 60 mV), duraquinone ($E'_{0,7}$ = 5 mV), menadione ($E'_{0,7}$ = 0 mV), plumbagin ($E'_{0,7}$ = -40 mV), phenazine methosulphate ($E'_{0,7}$ = -125 mV), 2-hydroxy-1,4-naphtoquinone ($E'_{0,7}$ = -152 mV) and anthraquinone sulfonate ($E'_{0,7}$ = -225 mV). A silver/silver chloride electrode previously calibrated in a saturated quinhydrone solution at pH 7 was used. The redox titration was performed recording the entire spectra from 350-700 nm. The experimental data was analysed using MATLAB (Mathworks, South Natick, MA) for Windows, and

fitted with as the sum of two one-electron Nernst curves, since there was no evidence for haem-haem interactions.

The cytochrome *c* domain protein was analysed by NMR and EPR spectroscopies. For the NMR studies, the protein was prepared in D₂O and the pH and ionic strength was adjusted with 10 mM phosphate buffer, pH 6.9. Reduction of the protein was achieved by addition of small volumes of a concentrated solution of sodium dithionite. 1D ¹H-NMR spectra (1k scans) were acquired in a Bruker Avance 500 MHz spectrometer using a QXI probe at 298 K. The residual water signal was saturated using a selective pulse of 500 ms. EPR spectra were obtained, at 15 K, on a Bruker EMX spectrometer, with an Oxford instruments continuous flow helium cryostat.

Western blot analysis. The antiserum against the cytochrome *c* domain of subunit II of the *D. vulgaris* oxygen reductase was produced by Centre Lago Company. To this end, 800 µg of the purified cytochrome *c* domain were applied in an SDS-PAGE gel and the protein band was cut and used for the immunization of rabbits. The serum of the rabbits final bleeding was utilized in the Western blot analysis.

D. vulgaris Hildenborough was grown anaerobically at 37 °C in sealed flasks (70 or 200 ml) in the following media: lactate/sulphate (L/S) (26); L/S modified by substituting lactate by 40 mM pyruvate (pyruvate/sulphate-P/S); L/S where lactate was replaced by 20 mM acetate (acetate/sulphate-A/S); L/S in which lactate was replaced by formate (40 mM) and acetate (20 mM) (formate/acetate/sulphate-Fo/A/S); L/S with lactate and sulphate substituted by succinate (28 mM) and fumarate (50 mM); (succinate/fumarate-Sc/Fu); and L/S with thiosulphate used instead of sulphate (40 mM) (lactate/thiosulphate-L/T). Cells were also grown under the following stress conditions: oxygen exposure – cells grown in L/S medium at an OD₆₀₀ of 0.6, were bubbled with O₂ gas for 3 h; iron stress – cells were grown under iron starvation conditions or iron excess by supplementation of the L/S medium

with 5 μ M or 60 μ M of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, respectively; nitrate and nitrite – cells were grown in L/S medium containing 25 mM of NaNO_3 ; cells in L/S were grown until an OD_{600} of 0.5 at which point 100 mM NaNO_3 or 2.5 mM NaNO_2 was added to the culture for 5 h and 2.5 h, respectively. The nitrate and nitrite concentrations were similar to those used in the transcriptomic study of *D. vulgaris* described in (27, 28). All cells were collected by centrifugation (7000 x g, 20 min), resuspended in buffer A and disrupted in a French Press. The protein content of the crude extracts was quantified by the bicinchoninic acid method and 30 μ g of total protein was applied into a 12 % SDS-PAGE gel. After electrophoresis the proteins were transferred to a nitrocellulose membrane (0.45 μ m) (Bio-Rad) in a trans-blot semi-dry transfer cell apparatus (Bio-Rad) (1 h, 15 V). The membrane was equilibrated in dry milk 5 % (w/v) dissolved in 10 mM Tris-HCl pH 7.5 with 150 mM NaCl (TBS-T buffer), followed by overnight incubation (4°C) with the antiserum raised against the cytochrome *c* domain of subunit II of the *D. vulgaris* oxygen reductase (1:2500). The membrane was then washed with TBS-T buffer and incubated at room temperature for 1 h with the anti-rabbit IgG alkaline phosphatase conjugate (1:7500) (Sigma). After a wash of the unbound anti-rabbit IgG with TBS-T buffer, a solution containing nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Fluka) was used for detection. The Western blot was also performed using the antiserum raised against the subunit I of *Paracoccus (Pa.) denitrificans aa3* (1:500) (gift from Prof. Bernd Ludwig). The pre-stained STD broad range molecular marker of Bio-Rad was used in the Western blots.

Membrane preparation and oxygen reduction assays. *D. vulgaris* Hildenborough cells were grown anaerobically in lactate/sulphate medium, at 37°C, in a 3-liter reactor (Applikon, Biocontroler 4DI 1030). After centrifugation, the cells were resuspended in buffer A plus 20 μ g/ml DNase and disrupted. For removal of unbroken cells, the extract was centrifuged at

5000 x *g* for 50 min and membranes were collected by centrifugation at 160000 x *g* for 2 h. After resuspension of the membrane fraction in buffer A, the protein content was determined. The oxygen reduction activity was determined in an Iso2 dissolved oxygen meter (World Precision Instruments, Inc). The assay was carried out at 30 °C using 2.7 mg of membranes and 0.8 mM *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) as artificial electron donor, that was kept reduced with 8 mM of sodium ascorbate. To test if the cytochrome c_{553} could act as electron donor for the *D. vulgaris* membranes, in the assay TMPD was replaced by 6.3 μ M of purified *D. vulgaris* cytochrome c_{553} . In all cases, inhibition of the oxygen reduction activity was achieved by the addition of 20 μ M of KCN.

2.3 - Results and discussion

Sequence analysis of *D. vulgaris* haem-copper oxygen reductase. In the genome of *D. vulgaris* Hildenborough, the gene cluster DVU1810-DVU1816 comprises the genes coding for a putative haem-copper oxygen reductase (Figure II-2.1) (21). This cluster is formed by six genes, encoding two hypothetical proteins (DVU1810, DVU1816), a protohaem IX farnesyltransferase (DVU1811), orthologs of subunits I (DVU1815) and II (DVU1812), characteristic of this superfamily, and orthologs of subunit III (DVU1814) and subunit IV (DVU1813).



Figure II-2.1- Gene cluster encoding the haem-copper oxygen reductase of *D. vulgaris*: DVU1810, DVU1816 encode hypothetical proteins; DVU1811 encodes a putative protohaem IX farnesyltransferase; DVU1815, DVU1812, DVU1814 and DVU1813 encode orthologs of haem-copper oxygen reductase subunits I, II, III and IV, respectively. The gene encoding cytochrome c_{553} (DVU1817) which is in the close vicinity of the cluster is also represented.

The sequence of subunit I lacks the helix VI glutamyl (Glu278, *Pa. denitrificans* numbering) at the end of the D- channel; instead, there is a tyrosyl and a seryl in helix VI, which allows to classify this enzyme as a Type A2 haem-copper oxygen reductase. All the other residues considered important for the D- and K-proton channels are conserved. Indeed, the higher amino acid sequence similarity is found with enzymes of the A2 subfamily, ranging from 24 to 67 % of identity and 39 to 83 % of similarity. The residues that bind the di-copper CuA centre in oxygen reductase enzymes are also conserved in the subunit II (19); however subunit II contains at the C-terminus two canonical motifs CxxCH for binding of two haems *c*, a situation so far not observed in any other A or B type enzymes.

The amino acid sequence of *D. vulgaris* subunit II was extensively compared with the haem-copper oxygen reductases available in the databases, and the higher similarities were found with *caa3* enzymes, namely those of subfamily A2. Dendograms performed with either subunit I or II yielded the same result, strongly suggesting that the *caa3* enzymes (so far found only in the genomes of *Desulfovibrio* species, namely in *D. vulgaris* Hildenborough, *D. vulgaris* DP4 and *D. desulfuricans*) evolved from the *caa3* oxygen reductases (Figure II-2.2). As observed for these latter enzymes, the cytochrome *caa3* domain bears no resemblances with the monohaem or dihaem subunits of Type C (*cbb3*) oxygen reductases.

A more restricted analysis was performed using only the cytochrome *c* domains of *caa3* and *caa3* enzymes. Remarkably, in the *caa3* enzymes, the two *c* domains are quite similar to each other (identities of ~ 44 %), as well as highly similar to the single cytochrome *c* domain of the *caa3* proteins (Figure II-2.3).

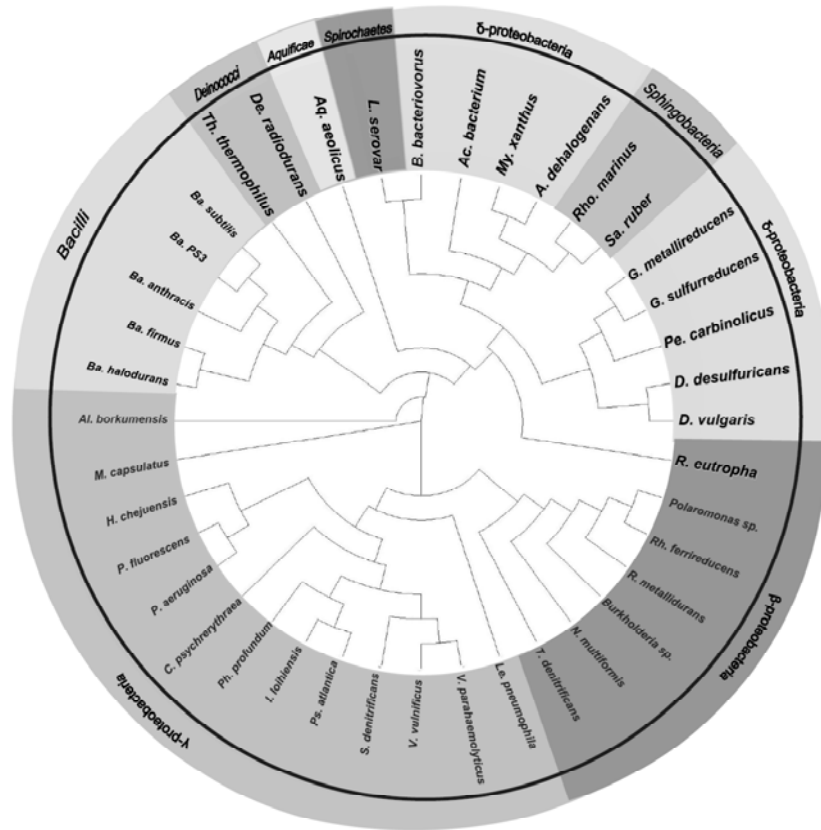


Figure II-2.2 - Dendrogram of the subunit II of *caa3* and *cca3* (*D. vulgaris* and *D. desulfuricans*) enzymes. The organism names (in bold letters) represent enzymes from the A2 subfamily. Organism and NCBI accession number: *D. (Desulfovibrio) vulgaris* (AAS96289.1), *D. desulfuricans* (ABB38623.1), *G. (Geobacter) metallireducens* (ABB30497.1), *G. sulfurreducens* (AAR33556.1), *Pe. (Pelobacter) carbinolicus* (ABA89767.1), *L. (Leptospira) serovar* (AAN47441.1), *B. (Bdellovibrio) bacteriovorus* (CAE77943.1), *Sa. (Salinibacter) ruber* (ABC44510.1), *A. (Anaeromyxobacter) dehalogenans* (ABC80578.1), *My. (Myxococcus) xanthus* (ABF90254.1), *Ac. (Acidobacteria) bacterium* (ABF41997.1), *R. (Ralstonia) eutropha* (AAZ62990.1), *Rh. (Rhodospirillum) ferrireducens* (ABD69410.1), *Polaromonas sp.* (ABE43196.1), *R. metallidurans* (ABF07147.1), *Burkholderia sp. 383* (ABB09770.1), *N. (Nitrosospora) multiformis* (ABB75074.1), *T. (Thiobacillus) denitrificans* (AAZ96278.1), *V. (Vibrio) vulnificus* (AAO07514.1), *V. parahaemolyticus* (BAC61879.1), *S. (Shewanella) denitrificans* (ABE56792.1), *Ph. (Photobacterium) profundum* (CAG18606.1), *I. (Idiomarina) loihiensis* (AAV81103.1), *Ps. (Pseudoalteromonas) atlantica* (ABG42745.1), *C. (Colwellia) psychrerythraea* (AAZ27294.1), *P. (Pseudomonas) fluorescens* (ABA71823.1), *P. aeruginosa* (AAG03495.1), *H. (Hahella) chejuensis* (ABC26969.1), *M. (Methylococcus) capsulatus* (AAU92995.1), *Le. (Legionella) pneumophila* (CAH14115.1), *Al. (Alcanivorax) borkumensis* (CAL17348.1), *Th. (Thermus) thermophilus* (AAA27484.1), *Aq. (Aquifex) aeolicus* VF5 (NP_214503.1), *De. (Deinococcus) radiodurans* R1 (NP_296338.1), *Rho. (Rhodothermus) marinus* (CAC08531.1), *Ba. (Bacillus), firmus* (AAA22364.1), *halodurans* (BAB06334.1), *sp. PS3* (BAA03045.1), *anthracis* (AAP27878.1), *subtilis* (CAB13362.1).

domain) was produced, purified and analysed. The cytochrome *c* domain protein migrated in SDS-PAGE with an apparent molecular mass of approximately 29 kDa, in accordance with the predicted mass plus the extra 6x-His tag tail. The UV-visible spectrum of the purified protein exhibits in the oxidized state a Soret band at 409 nm and a broad band between 500 and 600 nm (Figure II-2.4A). After reduction with sodium dithionite, the Soret band shifted to 416 nm and the β and α band appeared at 521 and 550 nm, respectively, the typical spectral profile for a reduced cytochrome *c* (Figure II-2.4A). The absorption maximum of the pyridine and redox pyridine hemochrome of the cytochrome *c* domain showed a band at 550 nm,

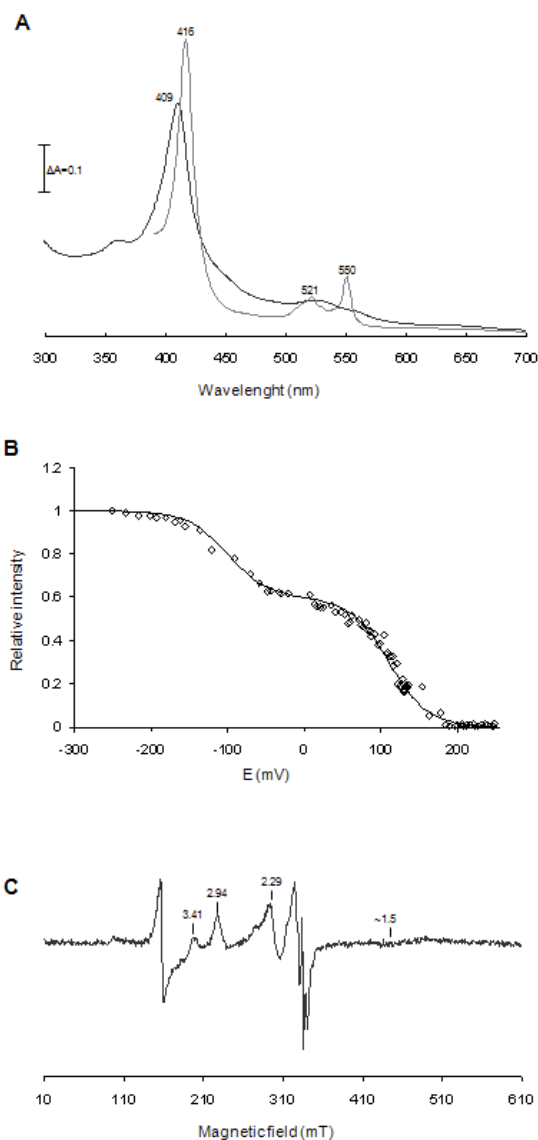


Figure II-2.4 - Characterization of the dihaem cytochrome *c* domain of subunit II haem-copper oxygen reductase of *D. vulgaris* Hildenborough. (A) UV-visible spectrum of the oxidized (black line) and reduced (dashed grey line) form of the cytochrome *c* domain. (B) Redox titration (monitored at the Soret band), fitted to the sum of two one- electron Nernst equations, with reduction potentials of -100 mV and +110 mV for the low-potential and high-potential haem and with a contribution of 0.4 and 0.6, respectively. (C) EPR spectrum of cytochrome *c* domain, at 10 K, 9.39 GHz and 2 mW.

confirming the presence of haem *c* (data not shown), with a ratio of 1.5 ± 0.2 haem per protein. The redox titration performed at pH 7.5, could be described as a sum of two one-electron Nernst equations, with reduction potentials of -100 mV for the low-potential haem and +110 mV for the high-potential haem, with relative contributions of 0.4 and 0.6, respectively (Figure II-2.4B). With such a large difference of reduction potentials possible homotropic haem-haem interactions are not observable. It should be stressed that these reduction potentials may not reflect the values in the intact protein, where the cytochrome domain will be sensing a different environment. The distinct contributions for the absorption data may result from slightly absorption coefficients of the two haems, but both show identical absorption maxima in the Soret and α bands.

In order to identify the ligands of the haems *c* the protein was analysed by NMR spectroscopy. The 1D ^1H -NMR spectrum of the oxidized form of the cytochrome *c* domain showed features characteristic of a protein containing low-spin paramagnetic haems. After reduction of the enzyme, the paramagnetic signals in the high frequency region disappear, and the spectrum showed the fingerprint of a typical diamagnetic haem protein that has a histidine-methionine coordinated haem, *i.e.*, it exhibits a signal in the low frequency region at -2.76 ppm that corresponds to the methionine ϵ methyl (data not shown) (31, 32). The EPR spectrum of the oxidized cytochrome *c* domain has *g*-values of 3.41, 2.94, 2.29, and ~ 1.5 confirming the presence of two haems *c* in the low spin state (Figure II-2.4C). Spectral simulations indicated that the two haems are present in a $\sim 1:1$ ratio, one with principal *g*-values of 2.94, 2.29 and 1.51 (rhombic ligand field) and the other of the “strong g_{max} ” type, with only the resonance at $g=3.41$ well observed (axial ligand field at the haem iron). The resonances at $g=4.3$ and $g\sim 2.0$ are due to minor amounts of contaminants.

Analysis of the expression of the *D. vulgaris* haem-copper oxygen reductase. The expression of *D. vulgaris* haem-copper oxygen reductase subunit II was analysed by Western blotting of crude extracts of cells grown under different conditions and using the antibody raised against the cytochrome *c* domain of subunit II of the enzyme. In all the conditions tested, expression of subunit II of the haem-copper oxygen reductase was detected by the development of a band around 54 kDa, which corresponds to the molecular mass of the complete subunit II.

D. vulgaris cells were grown with different electron donors or acceptors and submitted to different stresses. The electron donors used were lactate, pyruvate, acetate and formate/acetate, having sulphate as electron acceptor. It was also analysed the effect of thiosulphate as electron acceptor using lactate as electron donor, and that of succinate and fumarate replacing lactate and sulphate, respectively. The results showed that the expression of the haem-copper oxygen reductase in *D. vulgaris* cells was essentially not affected by the type of electron donor or acceptor used for the anaerobic metabolism (Figure II-2.5).

Nitrite was previously reported to inhibit sulphate reduction by *D. vulgaris* cells even in the presence of a sub-lethal concentration of nitrite, *i.e.* 2.5 mM (27). Our data showed that addition of nitrite did not alter the expression of the subunit II of the haem-copper oxygen reductase of *D. vulgaris* (Figure II-2.5). This result is in accordance with that obtained in transcriptional studies, where no significant difference in the gene expression level was observed upon exposure to similar conditions of nitrite stress (28). Likewise, the inclusion of nitrate in the medium in any of the concentrations tested did not led to a significant variation in the band intensities (Figure II-2.5).

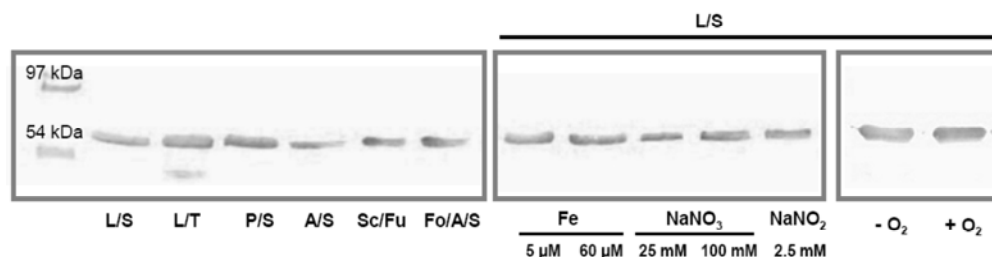


Figure II-2.5 - Expression analysis of subunit II oxygen reductase in cells of *D. vulgaris* grown under different conditions accessed by Western blot performed with an antiserum raised against the cytochrome *c* domain. The cell extracts were applied in three independent SDS-PAGE gels: L/S (lactate/sulphate); L/T (lactate/thiosulphate) P/S (pyruvate/sulphate); A/S (acetate/sulphate); Sc/Fu (succinate/fumarate); Fo/A/S (formate/acetate/sulphate); L/S supplemented with 5 or 60 μM of iron (Fe); L/S supplemented with 25 mM of nitrate (NaNO₃); L/S with 100 mM of nitrate (NaNO₃); L/S in the presence of 2.5 mM of nitrite (NaNO₂); growth in L/S and exposed O₂. The 54 and 97 kDa bands of the molecular weight marker are also displayed.

Since the oxygen and iron metabolism are linked (33) the influence of the iron concentration on the expression of *D. vulgaris* haem-copper oxygen reductase was also evaluated. It was observed that an excess or deficiency of iron did not affect the expression of the subunit II of the enzyme (Figure II-2.5).

Previous transcriptomic analysis of *D. vulgaris* showed that growth of cells in the presence of 0.1 % of oxygen causes a quite small increase in the transcription of the gene encoding subunit II of the haem-copper oxygen reductase (34). Accordingly, when we investigated the expression of the enzyme no meaningful difference was also observed between cells of *D. vulgaris* submitted to oxygen and those grown anaerobically (Figure II-2.5).

The expression of the *D. vulgaris* subunit I of haem-copper oxygen reductase was also analysed in cells grown under all the above-mentioned conditions, using the antibody against subunit I of the *aa₃* haem-copper oxygen reductase of *Pa. denitrificans*, which cross reacts with *D. vulgaris* subunit I. The results revealed that, as observed for subunit II, the expression of subunit I is independent of the different growth conditions tested (data not shown). Hence, the expression of the haem-copper oxygen reductase seems to be constitutive.

Cytochrome c_{553} gives electrons to membranes of *D. vulgaris*. *D. vulgaris* membranes were found to be able to reduce oxygen when accepting electrons from the artificial electron donor TMPD, with a TMPD:oxygen oxidoreductase activity of $12 \pm 4 \mu\text{mol O}_2.\text{min}^{-1}.\text{mg}^{-1}$ (Table II-2.1). Upon addition of KCN, a well known inhibitor of the haem-copper oxygen reductase family, the rate of oxygen reduction decreased by 35 %, providing evidence for the presence of an active haem-copper oxygen reductase in the membranes of *D. vulgaris*. Note that the addition of KCN up to 500 μM did not significantly change the percentage of inhibition (data not shown).

One of the possible electron carriers for the haem-copper oxygen reductase is the cytochrome c_{553} , encoded by the gene DVU1817, which is located in the vicinity of the gene cluster encoding the oxygen reductase of *D. vulgaris* (Figure II-2.1). This gene is also present in the vicinity of the genes for subunits of the haem-copper oxygen reductase in the genomes of *D. desulfuricans* G20, *D. vulgaris* DP4 and *D. vulgaris* Miyazaki (35). In order to assess if the cytochrome c_{553} of *D. vulgaris* was able to give electrons for the haem-copper enzyme, TMPD was replaced by the ascorbate-reduced cytochrome c_{553} that has a midpoint potential of +62 mV (36). The value of activity determined ($5 \pm 1 \mu\text{mol O}_2.\text{min}^{-1}.\text{mg}^{-1}$) indicated that the monohaem cytochrome c_{553} may serve as electron donor to *D. vulgaris* membranes during the oxygen reduction process. In this case it was also observed that addition of KCN caused partial inhibition of oxygen consumption (Table II-2.1).

Table II-2.1 -Oxygen reduction activities performed in the *D. vulgaris* membranes using TMPD or cytochrome c_{553} as electron donors.

	Activity ($\mu\text{mol O}_2.\text{min}^{-1}.\text{mg protein}^{-1}$)	KCN Inhibition (%)
TMPD:oxygen oxidoreductase	12 ± 3	35
Cytochrome c_{553} :oxygen oxidoreductase	5 ± 1	22

2.4 - Conclusion

In this work, we have shown that *D. vulgaris* Hildenborough contains a haem-copper oxygen reductase of the A2 type, which has the particularity, so far shared only with other *Desulfovibrio* species, of having a dihaem cytochrome *c* domain at the C-terminus of subunit II. The enzyme appears to be constitutive, under the various conditions tested, and is active. It was shown that the monohaem cytochrome α_{553} is able to transfer electrons to the membranes of *D. vulgaris*, enabling oxygen consumption. Although *Desulfovibrio* does not contain a quinol:cytochrome *c* oxidoreductase (*bci* complex), an ortholog for an alternative complex III, first identified in *Rho. marinus* at the biochemical level (37) and later at the genetic level (38), is present, as proposed previously by Yanyushin *et al.* (39).

The actual function of oxygen reductases in sulphate reducing bacteria remains to be clarified, since so far only *D. desulfuricans* ATCC 27774 cells were reported to sustain growth in the presence of oxygen (4). In fact, many enzymes from *Desulfovibrio* species, involved in sulphur and hydrogen metabolisms, among others, are extremely oxygen sensitive and proven to be reversibly or irreversibly inactivated by O₂, or even completely damaged by oxygen. Nevertheless, it was not yet proved if the main function of these enzymes is to contribute to energy conservation or if they are dedicated to fulfil a major role in oxygen detoxification, protecting the anaerobic metabolism of these bacteria against traces of oxygen present in the neighbouring environment and, at the same time, enabling energy conservation by oxidative phosphorylation.

2.5 - Acknowledgments

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Functional characterization of the early steps of the tetrapyrrole biosynthetic pathway in *Desulfovibrio vulgaris* Hildenborough[‡]

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Summary

The biosynthesis of the tetrapyrrole framework has been investigated in the sulphate reducing bacterium *Desulfovibrio vulgaris* Hildenborough by characterization of the enzymes required for the transformation of 5-aminolevulinic acid into sirohydrochlorin. Porphobilinogen synthase (HemB) was found to be a zinc-dependent enzyme that exists in its native state as a homohexamer. Porphobilinogen deaminase (HemC) was shown to contain the dipyrromethane cofactor. Uroporphyrinogen III synthase is found fused with a uroporphyrinogen III methyltransferase (HemD-CobA). Both activities could be demonstrated in this amalgamated protein and the individual enzyme activities were separated by dissecting the relevant gene to allow the production of two distinct proteins. A gene annotated in the genome as a bifunctional precorrin-2 dehydrogenase/sirohydrochlorin ferrochelatase was in fact shown to act only as a dehydrogenase and is simply capable of synthesizing sirohydrochlorin rather than sirohaem. Genome analysis also reveals a lack of any uroporphyrinogen III decarboxylase, an enzyme necessary for the classical route to haem synthesis. However, the genome does encode some predicted haem *d1* biosynthetic enzymes even though the bacterium does not

contain the *cdh* nitrite reductase. We suggest that sirohydrochlorin acts as a substrate for haem synthesis using a novel pathway that involves homologues of the *dh* biogenesis system. This explains why the uroporphyrinogen III synthase is found fused with the methyltransferase, bypassing the need for uroporphyrinogen III decarboxylase activity.

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3.1 - Introduction

Sulphate reducing bacteria (SRB) are environmentally important microorganisms that are found in a wide range of habitats but are reliant on being able to reduce sulphate in a dissimilatory manner to produce sulphide. To assist them in their metabolic challenge SRB employ a rich repertoire of redox groups including porphyrins and related modified tetrapyrroles. Genomic analysis suggests that SRB are able to make at least three types of modified tetrapyrroles, including haem, sirohaem and adenosylcobalamin, the coenzyme form of vitamin B₁₂.

Modified tetrapyrroles have a similar structural architecture reflecting the fact that they are all derived from a common macromolecular intermediate, uroporphyrinogen III. Thus all modified tetrapyrroles are made along a branched biosynthetic pathway, as outlined in Figure II-3.1. The pathway starts with the generation of 5-aminolevulinic acid¹ (ALA), the first common intermediate of tetrapyrrole biosynthesis, which is synthesized either from succinyl-coenzyme A and glycine (in the case of

some proteobacteria, fungi, yeast and animals) or from a tRNA-bound glutamate (in plants, algae, archaea and eubacteria) (1, 2). The next step involves the asymmetric condensation of two ALA molecules to form the monopyrrole porphobilinogen (PBG), in a Knorr-type condensation reaction catalysed by the enzyme porphobilinogen synthase (also called 5-aminolevulinic acid dehydratase), which is encoded by the *hemB* gene (4). Porphobilinogen synthases are distinguished according to their requirement in the active site for zinc or magnesium atoms and the need for magnesium

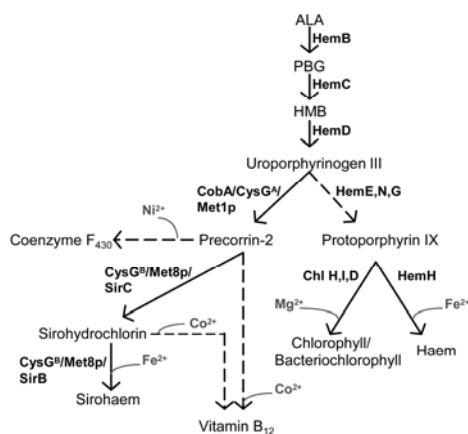


Figure II-3.1 - Tetrapyrrole metabolic pathway. Adapted from (3).

in an allosteric binding site. The only known porphobilinogen synthases where these metal binding sites are absent are from the bacterial genus *Rhodobacter* (5). *R. capsulatus* porphobilinogen synthase was shown not to require any metal ion for catalysis, having the added peculiarity of forming a hexameric structure (6), unlike most other studied porphobilinogen synthases that form octamers (eg. (7)).

Four molecules of the pyrrole PBG are subsequently polymerised into the linear tetrapyrrole hydroxymethylbilane (HMB) by the action of porphobilinogen deaminase, which is coded by *hemC*. The enzyme forms stable enzyme-intermediate complexes with one, two and three PBG-derived pyrrole molecules. A feature of this enzyme is the presence of a covalently bound dipyrromethane cofactor where the growing poly-pyrrole chain is assembled (8, 9). The HMB intermediate is further modified and cyclised by uroporphyrinogen III synthase (encoded by the *hemD* gene) to yield uroporphyrinogen III (10). In the absence of uroporphyrinogen III synthase, HMB cyclises spontaneously to yield the uroporphyrinogen I isomer (10). The synthase is able to invert the terminal pyrrole unit of HMB, ring D, and cyclise the macromolecule to give the asymmetric and only biologically relevant type III isomer. In many organisms uroporphyrinogen III represents the first branch point in the pathway, where the action of enzymes such as HemE, F (N) and G results in the formation of protoporphyrin IX, a precursor of modified tetrapyrroles such as haem and chlorophyll. Alternatively, uroporphyrinogen III can undergo an S-adenosyl-L-methionine (SAM) dependent-transmethylation at positions 2 and 7 (by CysG, Met1p or CobA depending on the organism) to generate precorrin-2, a highly unstable yellow dipyrrocorphin (11, 12). Precorrin-2 is further modified by a dehydrogenase (CysG, Met8p or SirC enzymes) to yield sirohydrochlorin. This isobacteriochlorin can be chelated with either ferrous iron to form sirohaem by the action of sirohydrochlorin ferrochelatase (CysG, Met8p or SirB enzymes) or with cobalt to generate cobalt-sirohydrochlorin, an

intermediate that is directed along the cobalamin biosynthetic pathway. Thus, as with uroporphyrinogen, sirohydrochlorin also represents an important branch point in the biosynthesis of modified tetrapyrroles.

In *E. coli* and *Salmonella enterica* the transformation of uroporphyrinogen III into sirohaem is performed by a single multifunctional enzyme, sirohaem synthase (CysG). The SAM-dependent methyltransferase activity is associated with the C-terminal domain of CysG (CysG^A) and the NAD⁺-dependent dehydrogenation and ferrochelation activities are linked to the N-terminal domain (CysG^B) (13, 14). In yeast, two independent enzymes, Met1p (SAM-dependent methyltransferase) and Met8p (NAD⁺-dependent dehydrogenase/ferrochelataase) are required for the sirohaem synthesis (15). A third alternative is found in *Bacillus megaterium*, where the three reactions are catalysed independently, by SirA (SAM-dependent uroporphyrinogen III methyltransferase), SirC (NAD⁺-dependent precorrin-2 dehydrogenase) and SirB (sirohydrochlorin ferrochelataase) (16).

Fused enzyme systems are not just restricted to the sirohaem branch of the pathway. In some bacterial species, such as *Selenomonas ruminantium* (17), *Lactobacillus reuteri* (18) and *Clostridium josui* (19), the uroporphyrinogen III methyltransferase and uroporphyrinogen III synthase activity is performed by one single protein, since the *cobA* and *hemD* genes are linked together. The biochemical logic and metabolic importance of such fusions is not fully understood.

The sulphate reducers of the *Desulfovibrio* genus have many different haemproteins (20-22) that play essential roles in the metabolism of the organism. Although tetrapyrrole biosynthesis has not been studied in any molecular detail in the SRB, earlier research on haem synthesis in *D. vulgaris* had uncovered one of the most interesting discoveries in tetrapyrrole biosynthesis of recent times as it demonstrated that haem is made by a novel route utilizing precorrin-2 or sirohydrochlorin as an intermediate (23). This new pathway is likely also to be the major route for

haem synthesis in the archaea. A follow-up study in *D. vulgaris* identified 12,18-didecarboxysirohydrochlorin (or its reduced form) as a possible intermediate but no specific enzymes/genes were linked with the metabolic activity. The authors also suggested that this novel pathway proceeded via coproporphyrinogen III, but this was not rigorously shown (23). Despite the novelty of this intriguing pathway, no genomic or biochemical investigation into tetrapyrrole synthesis in *D. vulgaris* has been undertaken even though the genome has been fully sequenced. In this paper we searched the *D. vulgaris* Hildenborough genome (24) and noted it contains genes encoding enzymes for the transformation of ALA into sirohydrochlorin via uroporphyrinogen III. Consistent with the presence of a novel haem biosynthetic pathway, the organism appears to be missing the majority of genes that encode the classical enzymes required for the transformation of uroporphyrinogen III into haem. In this paper we report the characterization of the early enzymes of tetrapyrrole assembly and modification in *D. vulgaris* and suggest enzymes that may be involved in the novel haem pathway.

3.2 - Materials and Methods

Cloning and expression of recombinant proteins. *D. vulgaris* Hildenborough genes encoding putative porphobilinogen synthase (*hemB*), porphobilinogen deaminase (*hemC*), uroporphyrinogen III methyltransferase/synthase (*cobA/hemD*), sirohaem synthase (*cysG^B*) were PCR amplified from genomic DNA using specifically designed oligonucleotides (Table II-3.1). Two additional oligonucleotides were designed for amplification of truncated forms of *cobA/hemD* to generate *hemD* or *cobA* encoding regions. The individual DNA fragments were cloned into pET vectors (Novagen) (Table II-3.1), which produced proteins with a 6x-His-Tag tail in the N-terminal or C-terminal, depending on the vector utilized. The genes *cobA/hemD*, *cobA* and *cysG^B* were also subcloned into pETac (25) for

use in complementation studies. The integrity of the gene sequence for all cloned fragments was confirmed by DNA sequencing.

Table II-3.1 - Oligonucleotides and plasmids used in this work.

Gene	Putative enzyme	Oligonucleotides	Plasmid
<i>hemB</i>	Porphobilinogen	5'-TCCATATGGGAGACTTCTTCAGAGG-3'	pET14b(+) <i>hemB</i>
	synthase	5'-TCACTAGTTTCAGCGCACCAGCCCTTTGGCGAGAAGC-3'	
<i>hemC</i>	Porphobilinogen	5'-TCCATATGAAGCACCTCGTCATCGCAACC-3'	pET14b(+) <i>hemC</i>
	deaminase	5'-TCACTAGTCTACTGCCTGTGCGGTGC-3'	
<i>cobA/hemD</i>	Uroporphyrinogen III	5'-GGAGGACATATGAAGGTCTATC-3'	pET22b(+) <i>cobA/hemD</i>
		5'-CCCGAGCTCGAGCAATTCAGG-3'	
	methyltransferase/	5'-TCCATATGAAGGTCTATCTCATCGGTGC-3'	pET14b(+) <i>cobA/hemD</i>
	synthase	5'-TCACTAGTGAGGTCTTACAATTCAGGCAATACG-3'	
<i>cobA^d</i>	Uroporphyrinogen III	5'-TCCATATGAAGGTCTATCTCATCGGTGC-3'	pET14b(+) <i>cobA^d</i>
	methyltransferase	5'-CTACTAGTTTAGTGCTCGAACCAGTT GAGACG-3'	
<i>hemD^d</i>	Uroporphyrinogen III	5'-TCCATATGAAGCCCTGCTGGGCAAGG-3'	pET14b(+) <i>hemD^d</i>
	synthase	5'-TCACTAGTGAGGTCTTACAATTCAGGCAATACG-3'	
<i>cysG^B</i>	sirohaem synthase, N-	5'-GCTATAAGAACCATATGCGACCC-3'	pET28a(+) <i>cysG^B</i>
	terminal	5'-GGCCGAAAGCTTGAATTCAGGC-3'	

Plasmids pET-28a(+)*cysG^B* and pET-22b(+)*cobA/hemD* were transformed into *E. coli* BL21Gold(DE3) cells (Stratagene) and plasmids pET-14b(+)*hemB*, pET-14b(+)*hemC*, pET-14b(+)*cobA* and pET-14b(+)*hemD* into *E. coli* BL21Star(DE3)(pLysS) cells (Invitrogen). In order to over express the proteins, the *E. coli* cells were grown at 37 °C, in Luria-Bertani (LB) medium with appropriate antibiotics, until an OD₆₀₀ between 0.5 and 0.8 was reached. Induction of the genes was achieved by addition of 200-400 µM of IPTG and by growing the cells for 4h at 37 °C, for *cobA/hemD* and *cysG^B* genes, or overnight at 19 °C for *hemB*, *hemC*, *cobA* and *hemD* genes. The truncated forms of *cobA/hemD* are designated *cobA^d* and *hemD^d* (where “d” stands for domain).

Protein purification. Cells expressing HemB, HemC, CobA/HemD, CobA^d (Met1-His246), HemD^d (Lys247-Lys503) and CysG^B proteins were harvested and resuspended in 20 mM Tris-HCl buffer, pH 8 containing 500

mM NaCl and 10 mM imidazole (buffer A); for the purification of CysG^B all buffers contained 5 % (v/v) glycerol. All cells were lysed by sonication and centrifuged for 20 minutes at $\sim 30000 \times g$. The soluble fraction was loaded on to a NiCl₂ charged chelating Sepharose resin (4 ml) (GE, Healthcare) equilibrated with buffer A. After washing with 5-10 volumes of buffer A containing 50 and 100 mM imidazole, all proteins were eluted with 2-5 volumes of buffer A with 500 mM imidazole, except CysG^B which was eluted with 100 mM imidazole. Protein fractions were pooled together after detection with Bio-Rad protein assay (BioRad), concentrated with Millipore Ultra Centrifugal Filters with a 10-kDa membrane and buffer exchanged, by means of a PD10 column (GE Healthcare), into buffer B (50 mM Tris-HCl pH 8), with the exception of CobA^d protein, which was buffer exchanged into buffer B containing 500 mM NaCl and 200 mM imidazole. The purity of the proteins was assessed by SDS-PAGE (26) and protein concentration was determined by the bicinchoninic acid method (27) using protein standards from Sigma. The native molecular mass of the various proteins was determined by gel filtration chromatography on either a Superdex 200 or 300 HR 10/30 column (GE, Healthcare), previously equilibrated with 20 mM Tris-HCl, pH 7.5.

The detection of the dipyrromethane cofactor of *D. vulgaris* porphobilinogen deaminase was determined by incubating 0.5 ml of the enzyme (0.2 mg) with an equivalent volume of Ehrlich's reagent (28) and by following the UV-visible spectral changes over 20 min.

Enzymatic assays. The various activity measurements were performed in a Hewlett Packard 8452A photodiode array spectrophotometer, a Shimadzu UV-1700 spectrophotometer or a BMG Labtech Flurostar Optima plate reader. All activities were assayed in buffer B, with the exception of porphobilinogen synthase which was assayed in 100 mM Tris-HCl pH 8.

Porphobilinogen synthase. HemB (0.5-5 μg) was pre-incubated for 5 min at 37 °C, and the reaction started by addition of ALA (5 mM), in a final volume of 0.5 ml. The reaction mixture was incubated for a further 5 min at 37 °C. The reaction was stopped by the addition of 500 μl of 10 % (w/v) TCA in 100 mM HgCl_2 . The mixture was centrifuged for 5 min at 9700 x g and an equal volume of a modified Ehrlich's reagent (28) was added to the supernatant. After a 15 min incubation, at room temperature, the absorbance at 555 nm was measured ($\epsilon_{555} = 6.02 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) (28).

Porphobilinogen deaminase. The HemC activity was determined essentially as described in (29), using 10-30 μg of enzyme and 200 nmol of PBG, and a $\epsilon_{405} = 5.48 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. One unit of enzyme is the amount of enzyme necessary to catalyse the utilization of 1 μmol of PBG in one hour.

Uroporphyrinogen III methyltransferase. Uroporphyrinogen III methyltransferase activity was followed by coupling it to precorrin-2 dehydrogenase (SirC) activity, allowing the transformation of uroporphyrinogen III into sirohydrochlorin ($\epsilon_{376} = 2.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) to be monitored. The substrate, uroporphyrinogen III, was generated anaerobically by incubating HemC and HemD from *B. megaterium* with 2 mg of PBG. The reaction mixture was prepared in an anaerobic chamber (Belle Technology, <2ppm O_2), in a final volume of 250 μl with NAD (100 μM), uroporphyrinogen III (2 μM), *B. megaterium* SirC (20 μg) and with different amounts of *D. vulgaris* bi-functional CobA/HemD (0.5-10 μg); the reaction was started by addition of SAM (100 μM).

Sirohaem synthase (CysG^B). Precorrin-2 dehydrogenase and sirohydrochlorin chelatase activities were assayed anaerobically following the formation of sirohydrochlorin or cobalt-sirohydrochlorin and sirohaem, respectively ($\epsilon_{376} = 2.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) (30). The substrates, precorrin-2 and sirohydrochlorin, were generated anaerobically as described previously (30). The dehydrogenase assay was performed in a 1 ml reaction, at 30 °C, with precorrin-2 (2 - 3 μM), NAD^+ (800 μM) and different amounts of *D. vulgaris*

CysG^B (0.5-10 µg). The chelatase activity was measured with 4.2 µM of sirohydrochlorin, 20 µM of Co²⁺ or Fe²⁺ and varying the amount of enzyme (10-50 µg).

In vitro generation of precorrin-2 with *D. vulgaris* enzymes. HemB, HemC and CobA/HemD enzymes of *D. vulgaris* were tested simultaneously and individually in a linked assay for the *in vitro* generation of precorrin-2 (31). The combination of enzymes and substrates used for each assay is described in Table II-3.2. All the reaction mixtures were performed in 50 mM Tris-HCl pH 8, in a final volume of 3 ml and contained between 0.05-5 mg of each enzyme, SAM (1 mg) and ALA (0.5 mg) or PBG (0.2 mg). The reaction was incubated for 2 h at room temperature, and after colour development UV-visible spectra were recorded. *B. megaterium* SirC (0.1 mg) was added to the assays to generate sirohydrochlorin, as a further confirmation of precorrin-2 production (Table II-3.2).

Table II-3.2- Enzymes and substrates utilized in the linked *in vitro* assays for anaerobic generation of precorrin-2

**Pseudomonas (P) denitrificans* CobA and *Bacillus (B) megaterium* HemC and HemD were used in the assays where *D. vulgaris* enzymes were tested individually.

<i>D. vulgaris</i> enzyme	Other enzymes and substrates present in the assay*	Precorrin-2 generation
HemB	<i>B. megaterium</i> HemC and HemD, <i>P. denitrificans</i> CobA, ALA, SAM	Yes
HemC	<i>B. megaterium</i> HemD, <i>P. denitrificans</i> CobA, PBG, SAM	Yes
HemD ^d	<i>B. megaterium</i> HemC, <i>P. denitrificans</i> CobA, PBG, SAM	Yes
Phe446Ser-HemD ^d	<i>B. megaterium</i> HemC, <i>P. denitrificans</i> CobA, PBG, SAM	No
CobA ^d	<i>B. megaterium</i> HemC and HemD, PBG, SAM	Yes
CobA/HemD	<i>B. megaterium</i> HemC, PBG, SAM	Yes
HemB,HemC,CobA/HemD	ALA, SAM	Yes
None	<i>B. megaterium</i> HemC and HemD, <i>P. denitrificans</i> CobA, PBG, SAM (Positive control)	Yes

Complementation of *E. coli cysG* and *E. coli hemD* mutant strains. The pETac plasmids containing the genes *cobA/hemD*, *cobA* and *cysG^B* were transformed into *E. coli cysG* mutant strain 302Δa (cysteine auxotrophic) (Table II-3.3).

Table II-3.3 - Strains and plasmids used in complementation studies together with *D. vulgaris* enzymes

*CobA activity tested with CobA/HemD protein and CobA^d. § HemD activity tested in the presence of CobA/HemD protein and HemD^d. † CysG^B protein was tested for precorrin-2 dehydrogenase and sirohydrochlorin ferrochelatase activity. ^Δ CysG^B protein was tested for sirohydrochlorin ferrochelatase activity. *Pseudomonas* (*P*); *Saccharomyces* (*Sa*); *Methanothermobacter* (*M*).

<i>D. vulgaris</i> enzyme	Strain	Plasmid	Plasmid description	References
CobA/HemD*	<i>E. coli</i> 302Δa	pETac- <i>cobA/hemD</i>	<i>D. vulgaris cobA/hemD</i> cloned in pETac	This study
		pCIQ- <i>metSp</i>	<i>Sa. metSp</i> cloned in pCIQ	This study
CobA ^d *	<i>E. coli</i> 302Δa	pETac- <i>cobA</i>	<i>D. vulgaris cobA</i> cloned in pETac	This study
		pCIQ- <i>metSp</i>	<i>Sa. cerevisiae metSp</i> cloned in pCIQ	This study
	<i>E. coli</i> 302Δa (Positive control)	pETac- <i>PdcobA</i> pCIQ- <i>metSp</i>	<i>P. denitrificans cobA</i> cloned in pETac <i>Sa. cerevisiae metSp</i> cloned in pCIQ	This study This study
CobA/HemD§	<i>E. coli</i> SASZ31	pET14b(+)- <i>cobA/hemD</i>	<i>D. vulgaris cobA/hemD</i> cloned in pET14b(+)	This study
HemD ^d §	<i>E. coli</i> SASZ31	pET14b(+)- <i>hemD</i>	<i>D. vulgaris hemD</i> cloned in pET14b(+)	This study
	<i>E. coli</i> SASZ31 (Positive control)	pET14b(+)- <i>EchemD</i>	<i>E. coli hemD</i> cloned in pET14b(+)	This study
CysG ^B †	<i>E. coli</i> 302Δa	pETac- <i>cysG^B</i>	<i>D. vulgaris cysG^B</i> cloned in pETac	This study
		pCIQ- <i>PdcobA</i>	<i>P. denitrificans cobA</i> cloned in pCIQ	(32)
	<i>E. coli</i> 302Δa (Positive control)	pETac- <i>metSp</i> pCIQ- <i>PdcobA</i>	<i>Sa. cerevisiae metSp</i> cloned in pETac <i>P. denitrificans cobA</i> cloned in pCIQ	(30) (32)
CysG ^B ^Δ	<i>E. coli</i> 302Δa	pETac- <i>cysG^B</i>	<i>D. vulgaris cysG^B</i> cloned in pETac	This study
		pCIQ- <i>sirCcobA</i>	<i>M. thermoautotrophicus sirC</i> and <i>P. denitrificans cobA</i> cloned in pCIQ	(32)
	<i>E. coli</i> 302Δa (Positive control)	pETac- <i>metSp</i> pCIQ- <i>sirCcobA</i>	<i>Sa. cerevisiae metSp</i> cloned in pETac <i>M. thermoautotrophicus sirC</i> and <i>P. denitrificans cobA</i> cloned in pCIQ	(30) (32)

E. coli cysG transformants were selected on LB plates, with 100 µg/ml ampicillin and 35 µg/ml chloramphenicol, and the mutant phenotype rescue tested on minimal medium plates with the appropriate antibiotics, in the absence and presence of cysteine. The plasmids pET14b(+)*cobA/hemD* and pET14b(+)*hemD* were introduced in *E. coli* SASZ31 (a *hemD* gene mutant strain) and selected on LB plates, with 100 µg/ml ampicillin by incubation at 37 °C, for approximately 20 h. The mutant strains were re-streaked on LB plates and incubated at 37 °C for a shorter time period (~12 h) to evaluate cell growth.

3.3 - Results and discussion

The genome of *D. vulgaris* (24) was searched using BLAST at NCBI, for homologs of known bacterial enzymes involved in the early steps of tetrapyrrole biosynthesis and modification, namely HemB, HemC, HemD, CobA and CysG. Four gene-derived amino acid sequences that have a high degree of sequence identity with these enzymes from different organisms, such as *E. coli* and *B. subtilis*, were identified (Table II-3.4). To study the *D. vulgaris* proteins, the corresponding genes were cloned and the recombinant proteins produced and biochemically characterized.

***D. vulgaris* porphobilinogen synthase is active as a hexamer.** A comparison of the gene deduced amino acid sequence of *D. vulgaris* porphobilinogen synthase with the porphobilinogen synthases of other organisms showed that *D. vulgaris* HemB contains the conserved cysteine rich sequence for Zn²⁺ binding (Cys121, Cys123 and Cys131, *D. vulgaris* HemB residue numbering), the determinant residues for allosteric magnesium binding (Arg11 and Glu238, *D. vulgaris* HemB residue numbering) (5) and the amino acid residues known to be involved in the binding of the substrate and reaction mechanism (34-36) (Figure II-3.2). The recombinant *D. vulgaris* HemB migrates by SDS-PAGE analysis with an

apparent molecular mass of ~36 kDa whereas gel filtration studies indicated the enzyme has a native mass of 226 kDa. This suggests that *D. vulgaris* exists as a homo hexamer. The specific activity of *D. vulgaris* HemB was determined in the presence of zinc to be 45 μmol of porphobilinogen $\text{h}^{-1}\text{mg}^{-1}$ with a K_M value for ALA of 0.05 mM (Table II-3.5). This activity is within the values measured for other bacterial porphobilinogen synthases such as *E. coli* (7), also assayed in the presence of zinc, and has a similar K_M .

	Identity (%)	Similarity (%)
<i>D. vulgaris</i> HemB		
<i>E. coli</i>	47	66
<i>Br. japonicum</i>	42	60
<i>P. aeruginosa</i>	44	62
<i>Pi. sativum</i>	41	54
<i>D. vulgaris</i> HemC		
<i>E. coli</i>	52	63
<i>S. enterica</i>	51	63
<i>R. capsulatus</i>	40	54
<i>C. josui</i>	33	50
<i>D. vulgaris</i> CobA/HemD		
<i>Se. ruminantium</i>	52	63
<i>L. reuteri</i>	29	47
<i>C. josui</i>	36	56
<i>Li. innocua</i>	35	53
<i>D. vulgaris</i> CobA ^d		
<i>P. denitrificans</i>	40	56
<i>B. megaterium</i>	48	67
<i>Pa. denitrificans</i>	34	47
<i>M. thermautotrophicus</i>	42	62
<i>D. vulgaris</i> HemD ^d		
<i>E. coli</i>	23	33
<i>S. enterica</i>	22	33
<i>B. megaterium</i>	27	47
<i>Pa. denitrificans</i>	16	27
<i>D. vulgaris</i> SirC		
<i>E. coli</i> CysG	12	20
<i>E. coli</i> CysG N-terminal	26	42
<i>Sa. cerevisiae</i> MET8	17	31
<i>B. megaterium</i> SirC	21	41

Table II-3.4 - Amino acid sequence identities and similarities of *D. vulgaris* enzymes with orthologs.

NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) accession numbers: HemB proteins: *D. vulgaris* (AAS95336), *E. coli* (NP_414903), *Bradyrhizobium* (*Br.*) *japonicum* (P45622), *Pseudomonas* (*P.*) *aeruginosa* (AAG08628), *Pisum* (*Pi.*) *sativum* (AAA33640); HemC proteins: *E. coli* (AAA67601), *Salmonella* (*S.*) *enterica* (CAD09382), *Rhodobacter* (*R.*) *capsulatus* (AAG50298), *Clostridium* (*C.*) *josui*, (BAA05861); CobA/HemD proteins: *Selenomonas* (*Se.*) *rumanium* (AAK00606), *Lactobacillus* (*L.*) *reuteri* (AAX14527), *C. josui* (BAA05862), *Listeria* *innocua* (NP_470501); CobA proteins: *P. denitrificans* (AAA25773), *Bacillus* (*B.*) *megaterium* (AAA22317), *Paracoccus* (*Pa.*) *denitrificans* (AAA93119), *Methanothermobacter* (*M.*) *thermautotrophicus* (NP_275310); HemD proteins: *E. coli* (NP_418248), *B. megaterium* (CAD48147), *Pa. denitrificans* (YP_915785), *S. enterica* (AAL22782); *E. coli* CysG and CysG N-terminal (NP_417827 and residues 1-223 of NP_417827), *Saccharomyces* (*Sa.*) *cerevisiae* MET8 (NP_009772), *B. megaterium* SirC (CAD48923). The amino acid sequence alignments were performed in Clustal W2 (33)

<i>D. vulgaris</i>	1	M	G	D	F	F	R	G	R	R	L	R	T	A	A	L	R	E	L	V	R	E	N	V	V	C	A	A	D	L	I	M	P	Y	F	V	V	E	T	D	E	P	A	F	R	K	E	I	G	A	50			
<i>E. coli</i>	2	T	D	L	I	Q	R	P	R	R	L	R	K	S	P	A	L	R	A	M	F	E	E	T	T	L	S	L	N	D	L	V	L	P	I	F	V	E	E	.	.	E	I	D	D	Y	K	A	V	E	A	49		
<i>Br. japonicum</i>	26	L	D	L	A	I	R	P	R	R	N	R	K	A	E	W	A	R	R	M	V	R	E	N	V	L	T	T	D	D	L	I	W	P	L	F	L	I	D	.	.	G	N	N	K	R	E	O	I	A	S	73		
<i>P. aeruginosa</i>	9	A	Y	P	Y	T	R	L	R	R	N	R	R	D	D	F	S	R	R	L	V	R	E	N	V	L	T	V	D	D	L	I	L	P	V	F	V	L	D	.	.	G	V	N	Q	R	E	S	I	P	S	56		
<i>Pi. sativum</i>	70	L	P	I	Q	R	R	P	R	R	N	R	S	P	A	L	R	S	A	F	Q	E	E	T	T	L	S	P	A	N	F	V	Y	P	L	F	I	H	E	.	.	G	E	E	D	.	T	P	I	G	A	116		
<i>D. vulgaris</i>	51	M	P	G	Q	F	Q	L	S	L	Q	E	.	L	E	K	Q	V	E	A	A	V	A	K	G	L	R	A	V	I	L	F	G	.	.	I	P	K	V	K	D	Y	K	G	S	E	A	Y	N	K	D	97		
<i>E. coli</i>	50	M	P	G	V	M	R	I	P	E	K	H	.	L	A	R	E	I	E	R	I	A	N	A	G	I	R	S	V	M	T	F	G	.	.	I	S	H	T	D	E	T	G	S	D	A	W	R	E	D	96			
<i>Br. japonicum</i>	74	M	P	G	V	E	R	L	S	V	D	Q	.	A	V	R	E	A	E	R	A	M	K	L	T	I	P	C	I	A	L	F	P	Y	T	D	P	S	L	R	D	E	E	G	S	E	A	C	N	P	N	122		
<i>P. aeruginosa</i>	57	M	P	G	V	E	R	L	S	I	D	Q	.	L	L	I	E	A	E	E	W	V	A	L	G	I	P	A	L	A	L	F	P	V	T	P	V	E	K	K	S	L	D	A	A	E	A	Y	N	P	E	105		
<i>Pi. sativum</i>	117	M	P	G	C	Y	R	L	G	W	R	H	G	L	L	E	E	V	A	K	A	R	D	V	G	V	N	S	V	V	L	F	P	K	I	P	D	A	L	K	T	P	T	G	D	E	A	Y	N	E	D	166		
<i>D. vulgaris</i>	98	G	I	V	Q	A	V	R	L	L	K	K	R	W	P	A	L	V	V	V	T	D	V	C	L	C	E	Y	T	D	H	G	H	C	G	L	V	R	Q	G	D	T	S	G	E	V	H	N	D	P	147			
<i>E. coli</i>	97	G	L	V	A	R	M	S	R	I	C	K	Q	T	V	P	E	M	I	V	M	S	D	T	C	F	C	E	Y	T	S	H	G	H	C	G	V	L	C	E	H	G	.	.	V	D	N	.	D	A	142			
<i>Br. japonicum</i>	123	N	L	V	C	Q	A	V	R	A	I	K	K	E	F	P	E	I	G	V	L	C	D	V	A	L	D	P	F	T	S	H	G	H	D	G	L	I	A	.	D	G	.	.	A	I	L	N	D	E	168			
<i>P. aeruginosa</i>	106	G	I	A	Q	R	A	T	R	A	L	R	E	R	F	P	E	L	G	I	I	T	D	V	A	L	D	P	F	T	T	H	G	Q	D	G	I	L	D	D	D	G	.	.	Y	V	L	N	D	V	152			
<i>Pi. sativum</i>	167	G	L	V	P	R	S	I	R	L	L	K	D	K	Y	P	D	L	I	I	Y	T	D	V	A	L	D	P	Y	S	S	D	G	H	D	G	I	V	R	E	D	G	.	.	V	I	M	N	D	E	213			
<i>D. vulgaris</i>	148	T	L	S	L	L	T	K	A	A	V	S	H	A	E	A	G	A	D	I	V	A	P	S	D	M	M	D	G	R	V	Q	A	I	R	A	G	L	D	A	N	G	F	S	H	I	P	V	M	S	Y	197		
<i>E. coli</i>	143	T	L	E	N	L	G	K	Q	A	V	V	A	A	A	A	G	A	D	F	I	A	P	S	A	A	M	D	G	Q	V	Q	A	I	R	Q	A	L	D	A	A	G	F	K	D	T	A	I	M	S	Y	192		
<i>Br. japonicum</i>	109	T	V	A	V	L	V	R	Q	A	L	V	Q	A	E	A	G	C	D	I	I	A	P	S	D	M	M	D	G	R	V	A	A	I	R	E	G	L	D	Q	A	G	L	I	D	V	Q	I	M	A	Y	218		
<i>P. aeruginosa</i>	153	S	I	D	V	L	V	R	Q	A	L	S	H	A	E	A	G	A	Q	V	V	A	P	S	D	M	M	D	G	R	I	G	A	I	R	E	A	L	E	S	A	G	H	T	N	V	R	I	M	A	Y	202		
<i>Pi. sativum</i>	214	T	V	H	Q	L	C	K	Q	A	V	A	Q	A	R	A	G	A	D	V	V	S	P	S	D	M	M	D	G	R	V	G	A	M	R	V	A	L	D	A	E	G	F	Q	H	V	S	I	M	S	Y	263		
<i>D. vulgaris</i>	198	A	V	K	Y	A	S	S	F	Y	G	P	F	R	E	A	A	E	S	T	P	Q	F	.	.	G	D	R	K	T	Y	Q	M	D	P	A	N	S	R	E	A	M	R	E	A	A	A	D	V	D	E	245		
<i>E. coli</i>	193	S	T	K	F	A	S	S	F	Y	G	P	F	R	E	A	A	G	S	A	L	K	.	.	G	D	R	K	S	Y	Q	M	N	P	M	N	R	R	E	A	I	R	E	S	L	D	E	A	Q	239				
<i>Br. japonicum</i>	219	A	A	K	Y	A	S	A	F	Y	G	P	F	R	D	A	I	G	S	A	K	T	L	T	.	.	G	D	K	R	T	Y	Q	M	D	S	A	N	T	D	E	A	L	R	E	V	E	L	D	I	S	E	267	
<i>P. aeruginosa</i>	203	S	A	K	Y	A	S	A	Y	Y	G	P	F	R	D	A	V	G	S	A	S	N	L	G	K	.	.	G	N	K	A	T	Y	Q	M	D	P	A	N	S	D	E	A	L	H	E	V	A	A	D	L	A	E	252
<i>Pi. sativum</i>	264	T	A	K	Y	A	S	S	F	Y	G	P	F	R	E	A	L	D	S	N	P	R	F	.	.	G	D	K	K	T	Y	Q	M	N	P	A	N	Y	R	E	A	L	T	E	M	R	E	D	E	S	E	311		
<i>D. vulgaris</i>	246	D	A	D	F	L	I	V	K	P	A	G	P	Y	L	D	I	I	R	Q	V	R	N	F	D	L	P	V	V	A	Y	Q	V	S	G	E	Y	S	M	I	R	A	A	G	I	N	G	W	I	N	295			
<i>E. coli</i>	240	G	A	D	C	L	M	V	K	P	A	G	A	Y	L	D	I	V	R	E	L	R	E	R	T	E	L	P	I	G	A	Y	Q	V	S	G	E	Y	A	M	I	K	F	A	A	L	A	G	A	I	D	289		
<i>Br. japonicum</i>	268	G	A	D	M	V	M	V	K	P	G	M	P	Y	L	D	I	V	R	R	V	K	D	T	F	A	M	P	T	F	A	Y	Q	V	S	G	E	Y	A	M	I	A	A	A	A	G	N	G	W	L	D	317		
<i>P. aeruginosa</i>	253	G	A	D	M	V	M	V	K	P	G	M	P	Y	L	D	I	V	R	R	V	K	D	E	F	R	A	P	T	F	V	Y	Q	V	S	G	E	Y	A	M	H	M	G	A	I	Q	N	G	W	L	A	302		
<i>Pi. sativum</i>	312	G	A	D	I	L	L	V	K	P	G	L	P	Y	L	D	I	I	R	L	L	R	D	N	S	P	L	P	I	A	A	Y	Q	V	S	G	E	Y	S	M	I	K	A	G	A	L	K	M	I	D	361			
<i>D. vulgaris</i>	296	E	E	A	V	I	M	E	S	L	.	L	G	M	K	R	A	G	A	D	L	I	I	T	Y	F	.	T	E	E	L	L	A	K	G	L	V	R	330			
<i>E. coli</i>	290	E	E	K	V	V	L	E	S	L	G	S	I	K	R	A	G	A	D	L	I	F	S	Y	F	.	A	L	D	L	A	E	K	K	I	L	R	324				
<i>Br. japonicum</i>	318	G	D	R	A	M	M	E	S	L	.	L	A	F	K	R	A	G	A	D	G	V	L	S	Y	F	.	A	P	K	A	A	E	K	L	R	T	Q	G	353						
<i>P. aeruginosa</i>	303	E	S	.	V	I	L	E	S	L	.	T	A	F	K	R	A	G	A	D	G	I	L	T	Y	F	.	A	K	Q	A	A	E	Q	L	R	R	G	337							
<i>Pi. sativum</i>	362	E	E	K	V	M	M	E	S	L	.	L	C	L	.	R	R	A	G	A	D	.	I	I	L	T	Y	F	.	A	L	Q	A	A	R	T	L	C	G	E	K	R	398							

Figure II-3.2 - Amino acid sequence alignment of porphobilinogen synthases from *D. vulgaris* (AAS95336), *E. coli* (NP_414903), *Bradyrhizobium* (*Br.*) *japonicum* (P45622), *Pseudomonas* (*P.*) *aeruginosa* (AAG08628) and *Pisum* (*Pi.*) *sativum* (AAA33640) (adapted from (34)) (NCBI accession numbers in parenthesis). Strictly conserved residues are shaded. The symbols represent functionally relevant residues in the *E. coli* enzyme (34, 35) which are also conserved in *D. vulgaris* porphobilinogen synthase: (#) cysteine residues that bind zinc; (*) residues involved in allosteric magnesium binding; (") arginine residues involved in binding A-side ALA; (—) residues of the lid region that covers the active site; (&") lysine residue required for correct function of the enzyme; (&) lysine residue that forms the Schiff base with P-side ALA; (\$) residues involved in binding of P-side ALA.

Table II-3.5- Kinetic parameters of *D. vulgaris* enzymes studied in this work

<i>D. vulgaris</i> enzyme	Specific Activity	K_M (substrate)
HemB	45 $\mu\text{mol. h}^{-1}. \text{mg}^{-1}$	0.05 mM (ALA)
HemC	20 $\mu\text{mol. h}^{-1}. \text{mg}^{-1}$	214 μM (PBG)
CobA/HemD	3 $\text{nmol. min}^{-1}. \text{mg}^{-1}$	0.4 μM (uroporphyrinogen III)
SirC	700 $\text{nmol. min}^{-1}. \text{mg}^{-1}$	70 μM (NAD ⁺)

Porphobilinogen deaminase of *D. vulgaris* contains the dipyrromethane cofactor in its active site. The purified recombinant *D. vulgaris* HemC migrates by SDS-PAGE with an apparent molecular mass of about 38 kDa, which is in close agreement with the predicted mass of 34 kDa. The UV-visible spectrum of the oxidized protein, at pH 8, contains two weak bands around 410 nm and 500 nm (data not shown), similar to the spectrum reported for the *E. coli* HemC enzyme (8). To investigate the presence of the dipyrromethane cofactor, the enzyme was mixed with Ehrlich's reagent, and the reaction followed by UV-visible spectroscopy over a 20 min period. A change in absorbance from 566 nm to 495 nm (Figure II-3.3) was observed, consistent with the presence of the dipyrromethane cofactor at the catalytic site of *D. vulgaris* HemC (8).

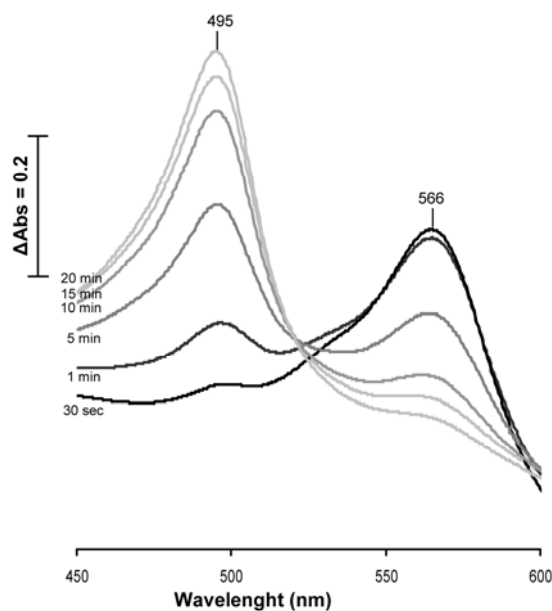


Figure II-3.3 - *D. vulgaris* porphobilinogen deaminase spectral features. Reaction with Ehrlich's reagent assessed by UV-visible. The spectra were recorded at the indicated times.

The specific activity of enzyme was measured as being 20 $\mu\text{mol h}^{-1}\text{mg}^{-1}$, which is similar to the activity of *E. coli* HemC enzyme (43 $\mu\text{mol h}^{-1}\text{mg}^{-1}$) (29). *D. vulgaris* HemC has a K_M of 214 μM for porphobilinogen, which is higher than the range of values measured for HemC of *E. coli*, *Rhodopseudomonas sphaeroides*, *Clostridium josui* and *Chlorella regularis* (19-89 μM) (19, 29, 37, 38).

In *D. vulgaris* the uroporphyrinogen III synthase and methyltransferase activities are performed by a single enzyme. A BLAST search with the locus DVU0734 revealed that the N-terminal domain of the protein (amino acids 1-246) exhibits significant amino acid sequence similarity with the uroporphyrinogen III methyltransferase enzymes (CobA), whereas the C-terminal region of DVU0734 (amino acids 247-503) has similarity with uroporphyrinogen III synthases (HemD). This suggests that in *D. vulgaris* the two activities are fused into a single multifunctional enzyme, which was named CobA/HemD. An amino acid sequence alignment of *D. vulgaris* DVU0734 with other separate bacterial CobA and HemD enzymes, allowed us to predict accurately the point of fusion between the two functional domains. Consequently, three proteins were recombinantly produced: the whole CobA/HemD protein, the truncated form corresponding to the N-terminal region (named CobA^d), and a second shorter protein corresponding to the C-terminal domain, (named HemD^d).

The initial characterization of DVU0734 was done by complementation studies. In *E. coli* the multifunctional enzyme CysG synthesizes sirohaem from uroporphyrinogen III. A deletion of this gene produces a mutant strain that is not able to produce sirohaem and thus cannot synthesize cysteine. The mutant phenotype can be rescued by complementation with the expression of genes from other organisms, which encode enzymes that catalyse the reactions performed by CysG (SAM-dependent methyltransferase of uroporphyrinogen III, NAD⁺-dependent

dehydrogenation of precorrin-2 and ferrochelation of sirohydrochlorin) (25, 31, 32). To test if the bi-functional CobA/HemD and CobA^d of *D. vulgaris* have uroporphyrinogen III methyltransferase activity *in vivo*, they were used to rescue the *E. coli cysG* mutant strain by co-expression with the bi-functional MET8 of yeast, which is a protein with precorrin-2 NAD⁺-dependent dehydrogenation and sirohydrochlorin ferrochelation activities (Table II-3.3). The *E. coli cysG* strain expressing MET8 of *Saccharomyces cerevisiae* and *cobA/hemD* or *cobA^d* of *D. vulgaris* was able to grow in the absence of cysteine, demonstrating that the *D. vulgaris* enzyme variants are able to perform the *in vivo* transmethylation of uroporphyrinogen III to yield precorrin-2.

The *in vivo* uroporphyrinogen III synthase activity of the *D. vulgaris* CobA/HemD and HemD^d variants was investigated by complementation of an *E. coli hemD* strain (SASZ31) (see (39)). This strain grows very poorly in LB medium, forming mini colonies, and thus complementation is observed only when the bacterium is transformed with a gene that allows the organism to form normal-sized colonies. Transformation of the mutant strain with plasmids harbouring *D. vulgaris cobA/hemD* or *hemD^d* resulted in fast growing strains, demonstrating that the *D. vulgaris cobA/hemD* eliminates the cell growth deficiency and indicating that the encoded protein is functioning *in vivo*. Furthermore, *E. coli* cells producing the *D. vulgaris* CobA/HemD exhibited a reddish pigmentation, which is due to the accumulation of sirohydrochlorin and trimethylpyrrocorphin and is consistent with increased uroporphyrinogen III methyltransferase activity (14, 40).

The *in vitro* activity of the recombinantly produced and purified enzyme variants was also studied. After purification, the CobA/HemD, which has a molecular mass of ~55 kDa, was tested for uroporphyrinogen III synthase and methyltransferase activity. A specific activity of 3 nmol.min⁻¹mg⁻¹ was determined for the uroporphyrinogen III methyltransferase activity of the

fused enzyme, with a K_M for uroporphyrinogen III of 0.4 μM (Table II-3.5). This is within the range of values observed previously, e.g. the *Pseudomonas denitrificans* CobA (1 μM) (41). In contrast to the *B. megaterium* and *P. denitrificans* CobAs (11, 41), the *D. vulgaris* CobA/HemD did not display substrate inhibition with uroporphyrinogen III, a characteristic that had previously been observed with an archaeal uroporphyrinogen III methyltransferase (42). The uroporphyrinogen III synthase activity of the CobA/HemD fusion protein was evaluated in a linked assay. Here, all the enzymes required to transform ALA into precorrin-2, except for the uroporphyrinogen III synthase, are mixed together. When ALA and SAM are added to this incubation the reaction stalls with the accumulation of HMB. If uroporphyrinogen III synthase is added, the reaction proceeds to the synthesis of precorrin-2 and the development of a characteristic yellow colour. When the CobA/HemD fusion was supplied to this assay (Table II-3.2), the generation of precorrin-2 was confirmed with the appearance of a characteristic UV-visible spectrum and the development of the typical yellow chromophore of the compound. The further addition of the *B. megaterium* SirC enzyme, which oxidizes precorrin-2, led to the formation of sirohydrochlorin providing further evidence for the formation of precorrin-2 in the reaction mixture (data not shown).

The activities of the two truncated proteins were investigated separately in an *in vitro* linked assay. This revealed that the CobA^d had uroporphyrinogen III methyltransferase activity but not uroporphyrinogen III synthase activity, while HemD^d was found to possess uroporphyrinogen III synthase activity (Table II-3.2). During the course of these studies a mutant variant, Phe446Ser, was serendipitously obtained in HemD^d and this variant showed no measurable uroporphyrinogen III synthase activity. The function of Phe446 in HemD enzymes is unknown, and although is not strictly conserved this residue seems to play an essential role. However, more data will be required to confirm the catalytic role of Phe446.

The *D. vulgaris* CysG^B has precorrin-2 dehydrogenase activity. The *D. vulgaris* CysG^B has 17, 21 and 26 % sequence identity with *Saccharomyces cerevisiae* MET8, *B. megaterium* SirC and to the N-terminal part of the *E. coli* CysG, respectively (Table II-3.4). Although this is a relatively low value of amino acid identity, it is in the same range of identity observed between the first 223 amino acids of *E. coli* CysG and MET8 (16 %). A common feature of all of these proteins is the presence of a consensus NAD⁺ binding motif at the start of the N-terminal region (43). This motif on the *D. vulgaris* CysG^B (GxGxxGx₁₀G) is identical to the one observed in MET8 (15) and similar to the one found in the N-terminal domain of the *B. megaterium* SirC (GxGxxAx₁₀G) and *E. coli* CysG (GxGxxAx₃Ax₆G).

CysG^B of *D. vulgaris* was assayed for NAD⁺-dependent precorrin-2 dehydrogenase activity by following the appearance of sirohydrochlorin, and was found to have a specific activity of ~700 nmol.min⁻¹.mg⁻¹ and a *K_M* for NAD⁺ of 70 μM (Table II-3.5). The specific activity of *D. vulgaris* CysG^B is significantly higher than the NAD⁺-dependent precorrin-2 dehydrogenase activity reported for the *B. megaterium* SirC (60 nmol.min⁻¹.mg⁻¹) (16). However, the *D. vulgaris* enzyme is not able to perform the insertion of iron or cobalt into sirohydrochlorin, a result that is consistent with complementation studies, where the corresponding gene was unable to rescue the *E. coli cysG* mutant strain phenotype (Table II-3.3). Cumulatively, these results show that the putative CysG^B of *D. vulgaris*, although annotated in the *D. vulgaris* genome as an orthologue of the yeast bi-functional enzyme Met8p (31), is in fact a functional SirC enzyme, a single NAD⁺-dependent precorrin-2 dehydrogenase that does not possess any chelatase activity (16).

***D. vulgaris* enzymes generate precorrin-2 from ALA in vitro.** An *in vitro* incubation with purified recombinant *D. vulgaris* HemB, HemC and CobA/HemD together with ALA and SAM resulted in the generation of

precorrin-2 (Figure II-3.4 and Table II-3.2). The presence of the yellow dipyrrocorphin was confirmed by the loading of purified *B. megaterium* SirC and NAD⁺ to the incubation, an addition that generated the isobacteriochlorin, sirohydrochlorin (Figure II-3.4).

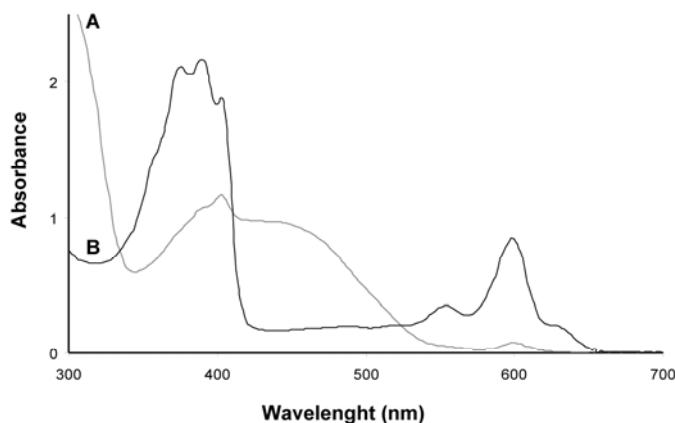


Figure II-3.4 - UV-visible spectra of precorrin-2 (A) and sirohydrochlorin (B) generated in the linked assay performed with *D. vulgaris* recombinant enzymes.

3.4 - Conclusion

***D. vulgaris* makes sirohydrochlorin.** In the work reported herein, the biochemical and functional characterization of several enzymes that catalyse the early steps of the modified tetrapyrrole biosynthetic pathway in *D. vulgaris* was undertaken. Thus, the *D. vulgaris* porphobilinogen synthase (HemB) was produced recombinantly and shown to be a zinc-dependent enzyme that appears to have a native hexameric structure, in contrast to the majority of bacterial HemB enzymes that are octamers. For porphobilinogen deaminase (HemC), the presence of the dipyrromethane cofactor, which covalently binds the growing poly-pyrrole chain, further supports the universal nature of this essential prosthetic group.

The recombinant *D. vulgaris* CobA/HemD was shown to be active both *in vivo* and *in vitro*, and represents the first characterisation of a bi-functional uroporphyrinogen III synthase/methyltransferase. This suggests that, in this organism, uroporphyrinogen III is not released as a free

intermediate. Rather, uroporphyrinogen III seems to be directed towards the formation of precorrin-2. This highly unstable intermediate is acted upon by a SirC-like protein, which, in the presence of NAD⁺, transforms it into sirohydrochlorin. It is known that sirohydrochlorin acts as an intermediate for B₁₂ biosynthesis, since it is chelated with cobalt to generate cobalt-sirohydrochlorin by CbiK (32). It also plays a role in sirohaem synthesis where ferrous chelation generates this prosthetic group, which is required by sulfite reductase and which is also found in *D. vulgaris*. However, it is also likely that sirohydrochlorin acts as an intermediate in the biosynthesis of haem.

***D. vulgaris* does not contain HemE, N, G or H.** One of the interesting features of the *D. vulgaris* genome is the absence of the genes that encode enzymes for the classical synthesis of haem from uroporphyrinogen III. Thus, there are no homologues to *hemE*, *hemN*, *hemG* or *hemH*, which encode the enzymes uroporphyrinogen III decarboxylase, coproporphyrinogen III oxidase, protoporphyrinogen IX oxidase and protoporphyrin IX ferrochelatase, respectively. The absence of these genes is consistent with the presence of a novel pathway for the transformation of precorrin-2 or sirohydrochlorin into haem. Previous work by Sano and co-workers had shown that haem must be made via precorrin-2, since the two methyl groups found on rings A and B of the final porphyrin product are derived from methionine and not from the C2 position of ALA (23). However, in their follow-up work, it was reported that an incubation of crude cell extracts of *D. vulgaris* with uroporphyrinogen III resulted in the appearance of sirohydrochlorin, 12,18-didecarboxysirohydrochlorin, coproporphyrin III and protoporphyrin IX. However, in this study only the structure of the novel 12,18-didecarboxysirohydrochlorin was fully characterised (23).

If coproporphyrinogen III is an intermediate, then it could be acted upon by an anaerobic coproporphyrinogen oxidase such as HemN. Interestingly,

the *D. vulgaris* genome does encode an orthologue of the radical SAM-reliant coproporphyrinogen III oxidase. The HemN proteins are characterised by the presence of a Fe-S redox group, which is constituted by 4 cysteines within the conserved motif (CxxxCxxCxC). However, in the *D. vulgaris* homologue, one of the cysteines is missing, suggesting that this *D. vulgaris* protein is not a HemN. Proteins missing this conserved motif have been shown not to be involved in haem biosynthesis (44). Thus, if coproporphyrinogen III is an intermediate, it is not clear how it is converted into protoporphyrin IX as there are no recognized enzymes present to undertake the required reactions.

Possible proteins involved in the novel haem synthesis pathway. A scan of the genome reveals a number of genes encoding enzymes that are designated for the synthesis of haem *d_l* (*nirD* and *nirJ*), the cofactor of the nitrite reductase *cdh*. However, *D. vulgaris* does not seem to have *cdh*. These haem *d_l* synthetic homologues are also found in some genomes of methanogens and other bacteria that do not have *cdh*. In the methanogens, it has also been shown that haem is also made via a dimethylated uroporphyrinogen III derivative such as precorrin-2 or sirohydrochlorin (45). Significantly, there are some mechanistic similarities between the pathways for haem *d_l* synthesis and haem construction via precorrin-2. In haem *d_l* synthesis the two propionic acid side chains attached at C3 and C8 of the macrocycle are lost and are replaced by oxygen. To achieve haem synthesis from precorrin-2/sirohydrochlorin, the acetic acid side chains at C2 and C7 have to be removed. Both processes may involve radical chemistry and thus could be mediated via a radical SAM enzyme system. It is therefore of interest to note that NirJ is proposed to be a radical SAM protein. We thus propose that the *D. vulgaris nirJ* and *nirD* genes are involved in the transformation of either sirohydrochlorin or sirohaem into haem.

In summary, the research herein described outlines how the basic tetrapyrrole framework is synthesized up to the first branch point, which we believe to be sirohydrochlorin. Genomic analysis thus suggests that cobalamin and sirohaem are made by classical previously described routes but that haem is synthesized along a novel route using homologues of the haem *d1* biosynthetic apparatus. The presence of a bifunctional CobA/HemD is consistent with uroporphyrinogen III not being a branch point in this bifurcated pathway and may represent a useful marker for an alternative haem biosynthetic route. More research is required to elucidate this surrogate synthesis. It is not clear why some of the *Desulfovibrio* or archaea have an alternative haem biosynthetic pathway. Possibly, the pathway evolved as a method of producing the more oxidized porphyrin ring system under anaerobic conditions at a time when the major tetrapyrroles were sirohaem and cobalamin. Then as molecular oxygen became more abundant so the haem biosynthesis pathway involving several oxidative steps in the synthesis of protoporphyrin evolved and became the more common route.

3.5 - Acknowledgments

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Two distinct roles for two functional cobaltochelatases (CbiK) in *Desulfovibrio vulgaris* Hildenborough*

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Summary

The sulphate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough possesses a large number of porphyrin-containing proteins whose biosynthesis is poorly characterized. In this work we have studied two putative CbiK cobaltochelatases present in the genome of *D. vulgaris*. The assays revealed that both enzymes insert cobalt and iron into sirohydrochlorin, being the specific activities with iron lower than that measured with cobalt. Nevertheless, the two *D. vulgaris* chelatases complement the *E. coli cysG* mutant strain showing that, *in vivo*, they are able to load iron into sirohydrochlorin. The results showed that the functional cobaltochelatases have distinct roles with one, CbiK^C, likely to be the enzyme associated with cytoplasmic cobalamin biosynthesis while the other, CbiK^P, is periplasmic located and possible associated with the iron transporter system. Finally, the ability of *D. vulgaris* to produce vitamin B₁₂ was also demonstrated in this work.

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4.1 - Introduction

Modified tetrapyrroles such as haems, sirohaem, and cobalamin (vitamin B₁₂) are characterised by a large molecular ring structure with a centrally chelated metal ion. This family of compounds share a common pathway until the formation of the first macrocyclic intermediate, uroporphyrinogen III, at which point the pathway branches. Each branch contains a unique chelatase that performs the insertion of a specific metal ion into the modified tetrapyrrole ring (1, 2). In haem biosynthesis, for example, ferrous iron is inserted into the protohaem precursor protoporphyrin IX by the protoporphyrin IX ferrochelatase (3). In the case of vitamin B₁₂ biosynthesis, cobalt is inserted into the macrocycle by a cobaltochelatase, which is either specific to the aerobic (oxygen-dependent) or anaerobic pathway for cobalamin biosynthesis (2). Along the aerobic pathway, precorrin-2 undergoes several enzymatic modifications that result in the formation of hydrogenobyrrinic acid *a,c*-diamide where cobalt is inserted by the class I ATP-dependent cobaltochelatase CobN-S-T (4, 5). Along the anaerobic pathway, cobalt insertion occurs at an early stage, at the level of sirohydrochlorin (6) by the class II ATP-independent cobaltochelatases CbiX or CbiK (1, 5, 7). The CbiX protein was first identified as a 320 amino acid enzyme, the so called long form (CbiX^L), whereas the archaeal ortholog is constituted by only around 110-145 amino acids, the short form (CbiX^S). The N-terminal and C-terminal domains of the long form of CbiX^L share a high degree of amino acid sequence similarity. CbiKs are formed by ~300 amino acid residues and share a low level of similarity with CbiX^L. Analysis of circular dichroism (CD) spectra suggested that the cobaltochelatases CbiK, CbiX^L and CbiX^S may have a similar overall topology (5). The X-ray structure determination of the CbiK from *Salmonella enterica* revealed also that the enzyme is highly similar with the protoporphyrin IX ferrochelatase from *Bacillus subtilis* (8, 9). More recent structural work on

CbiX^s has confirmed that this protein shares the same basic protein architecture as found in CbiK (10).

Sulfate reducing bacteria (SRB), belonging to the genus *Desulfovibrio*, are considered to be early organisms on the evolutionary scale and contain a very large number of proteins with modified porphyrins, some of them unusual. Examples of this include the rubredoxin oxygen oxidoreductase from *D. gigas* that contains iron uroporphyrin I (11) and bacterioferritin of *D. desulfuricans* ATCC 27774 which has an iron-coproporphyrin III cofactor (12). Proteins containing cobalt-porphyrin were also reported for *D. gigas* and *D. desulfuricans* (Norway) (13, 14), and the nature of the cobalt-porphyrin macrocycle was identified as Co^{III}-syrohydrochlorin (15). Studies in *D. vulgaris* suggested that in these organisms an alternative pathway for the biosynthesis of haem may be operative, since coproporphyrinogen III is formed not directly from uroporphyrinogen III but from the vitamin B₁₂ precursor, precorrin-2 (16). However, the biosynthesis of porphyrins in *Desulfovibrio* sp. remains poorly understood.

The genome of *D. vulgaris* Hildenborough encodes two putative cobaltochelataes, DVU0650 and DVU1365, that share a significant degree of amino acid sequence identity with *S. enterica* CbiK. In order to clarify the role of these proteins and their involvement in the modified tetrapyrrole biosynthesis in a *Desulfovibrio* sp., the genes were cloned, the proteins produced recombinantly and the isolated enzymes characterized.

4.2 - Materials and Methods

Cloning and expression of recombinant cobaltochelataes. The putative cobaltochelataes encoded by the genes DVU0650 and DVU1365 have 894 bp and 849 bp, respectively. Amplification of the two genes was achieved in PCR reactions using genomic DNA of *Desulfovibrio vulgaris* Hildenborough and

appropriated oligonucleotides specifically designed for each case. A second DNA fragment of the DVU0650 gene was also amplified in order to construct a truncated protein that starts at amino acid 29 (Δ 28DVU0650). The various DNA fragments were cloned into pET-28a(+) (Novagen) giving pET-28a(+)-DVU0650, pET-28a(+)- Δ 28DVU0650 and pET-28a(+)-DVU1365 allowing the encoded proteins, DVU0650, Δ 28DVU0650 and DVU1365, to be produced with an 6x-His-tag in the N-terminal region. Sequencing of the PCR products guaranteed the integrity of all gene sequences. To produce DVU0650 and Δ 28DVU0650 proteins, the recombinant plasmids were transformed in *Escherichia coli* BL21Gold(DE3) (Stratagene) and the cells were grown, at 30 °C, in Luria-Bertani (LB) medium containing kanamycin (30 µg/ml) until an OD₆₀₀=0.3. At this point, 200 µM of IPTG (isopropyl-β-D-thiogalactopyranoside) was added and the medium supplemented with 50 µM 5-aminolevulinic acid (ALA) and 100 µM FeSO₄. The culture was then grown overnight, at 15 °C. The overexpression of DVU1365 was achieved by transforming the recombinant plasmid in the BL21Gold(DE3) and growing the cells, at 37 °C, in LB medium containing 30 µg/ml kanamycin until an OD₆₀₀ of 0.8, followed by the addition of 200 µM of IPTG and 100 µM FeSO₄. The cells were then grown at 37 °C for 4h.

Protein purification and characterisation. Cells expressing DVU0650, Δ 28DVU0650 and DVU1365 proteins were harvested, resuspended in 20 mM Tris-HCl buffer, pH 7.5 (buffer A) with 20 µg/ml DNase and disrupted in a French Press. The soluble fraction was separated from the membranes by ultra centrifugation for 2 hours at 160000 x *g*. All purification steps were performed at 4°C, either in aerobic or anaerobic conditions.

DVU0650 protein was purified under aerobic and anaerobic conditions. The fraction isolated from the aerobic purification was used for the biochemical and spectroscopic characterisation. For the aerobic purification of DVU0650 protein, the soluble fraction from *E. coli* cells expressing

DVU0650 was applied onto a Chelating Sepharose fast flow column (130 mL) (GE, Healthcare), previously charged with NiCl_2 and equilibrated with buffer A containing 400 mM NaCl. Eleven volumes of a linear gradient up to 250 mM imidazole were applied to the column and the recombinant protein was eluted at 250 mM imidazole. After dialysis against buffer A, the protein fraction was loaded onto a Q-Sepharose High-Performance column (20 mL) (GE, Healthcare), previously equilibrated with buffer A. Ten volumes of a linear gradient up to 400 mM NaCl followed by four volumes of a linear gradient from 400 mM up to 1 M NaCl was applied and the protein was eluted with ~200 mM NaCl

DVU0650, $\Delta 28$ DVU0650 and DVU1365 proteins were purified under anaerobic conditions. The anaerobic purification steps were performed in a Coy model A-2463 anaerobic chamber filled with a gas mixture of 95 % argon plus 5 % hydrogen. The soluble fraction from the cells expressing DVU0650, $\Delta 28$ DVU0650 and DVU1365 was applied onto a HiTrap Chelating HP column (GE, Healthcare). The recombinant DVU0650 and $\Delta 28$ DVU0650 proteins were eluted with 5 volumes of buffer A containing 0.5 M NaCl and 200 mM imidazole, while the DVU1365 protein was eluted with 5 volumes of buffer A plus 250 mM imidazole. The protein fractions containing DVU0650 or $\Delta 28$ DVU0650 were passed through a PD10 desalting column (GE, Healthcare) in order to exchange the elution buffer to the 50 mM Tris-HCl buffer, pH 8 with 100 mM NaCl, while the DVU1365 protein fraction was dialyzed overnight against 50 mM Tris-HCl buffer, pH 8 with 8 % glycerol.

The purity of the proteins was analysed by SDS-PAGE gel (17) and the protein concentration was determined by the bicinchoninic acid method (18) using protein standards from Sigma. Haem content was assayed by the hemochromopyridine method (19) and haem extraction was performed according to the method described by Lubben *et al.* (20). The protein molecular mass was determined by gel filtration in a Superdex 200 column

according to the instructions of the manufacturer (GE, Healthcare), using commercially available standards from GE Healthcare.

Comparisons of the primary amino acid sequences were done using ClustalX (21) and the prediction of sorting signals was achieved using the SOSUI signal program (22, 23). UV-visible spectra were recorded at room temperature in a Shimadzu UV-1700 spectrophotometer with 4.5 μ M of protein and sodium dithionite was used as a reductant. The redox titration was performed at 25°C, under continuous agitation and argon atmosphere using 2.8 μ M of purified protein and 12 μ M of mediators, diluted in 50 mM Tris-HCl pH 7.5. The redox mediators used allowed to cover the interval range between +340 mV and -225 mV: N,N-dimethyl-p-phenylene-diamine (E'_0 = 340 mV), p-benzoquinone (E'_0 = 240 mV), 1,2-naphtoquinone-4-sulfonic acid (E'_0 = 215 mV), 1,2-naphtoquinone (E'_0 = 180 mV), trimethylhydroquinone (E'_0 = 115 mV), phenazine methosulfate (E'_0 = 80 mV), 1,4-naptoquinone (E'_0 = 60 mV), duroquinone (E'_0 = 5 mV), menadione (E'_0 = 0 mV), plumbagin (E'_0 = -40 mV), phenazine (E'_0 = -125 mV), 2-hydroxy-1,4-naphtoquinone (E'_0 = -152 mV), anthraquinone sulfonate (E'_0 = -225 mV). A silver/silver chloride electrode, previous calibrated in a saturated quinhydrone solution was used. The titration was performed from the oxidized to the reduced state and an experimental curve was obtained from the potential measured at 560 nm. The experimental data was analyzed using MATLAB (Mathworks, South Natick, MA) for Windows.

Concentrated fractions of DVU0650 (95 μ M) were prepared for EPR spectroscopy and the spectra were acquired at 15 K, on a Bruker ESP 308 spectrometer equipped with an Oxford Instruments continuous flow helium cryostat. The NMR sample was prepared in D₂O and the pH and ionic strength were set using 10 mM phosphate buffer, pH 6.9. 1D ¹H-NMR spectra (1k scans) were acquired, at 298 K, in a Bruker Avance 500 spectrometer using a QXI probe. The residual water signal was saturated

using a selective pulse of 500 ms. The sample was reduced by adding the appropriated volume of a saturated solution of sodium dithionite.

Activity assays. The chelatase activity assays were performed with proteins purified under either aerobic/anaerobic conditions and the activity was measured anaerobically following the formation of cobalt-sirohydrochlorin or sirohaem, by the decrease of absorbance at 376 nm and using the extinction coefficient $2.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ (24). The plasmid pETcoco-2ABCD (25) harbouring the genes encoding for uroporphyrinogen III methyltransferase (CobA) of *Methanosarcina barkeri*, porphobilinogen synthase (HemB) and precorrin-2 dehydrogenase (SirC) of *Methanothermobacter thermautotrophicus* and porphobilinogen (PBG) deaminase (HemC) and uroporphyrinogen III synthase (HemD) of *Bacillus megaterium* was introduced in *E. coli* BL21(DE3)pLysS (Novagen) and the proteins were overexpressed in order to produce sirohydrochlorin. To this end, the cells were grown overnight in LB medium containing ampicillin (50 µg/ml), chloramphenicol (34 µg/ml) and 0.2 % (w/v) glucose. The cells were then inoculated in LB medium with the same antibiotics described and grown at 37° C, until an OD600 ~0.5, followed by the addition of 0.02 % (w/v) of L-arabinose. After 2 h, 400 µM of IPTG were added and the cells were grown overnight at 24 °C. After breaking the cells in a French Press, the lysate was transferred to the anaerobic chamber where 2 ml of lysate was incubated with 2 mg SAM (S-adenosyl-L-methionine), 1 mg ALA and 1 mg NAD⁺, in a total volume of 6 ml in 50 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl. The pH of the reaction mixture was adjusted to 8 with 2 M KOH. After overnight incubation, at room temperature, the reaction mixture was filtered (0.22 µm filter) and passed through a DEAE-resin (Sigma) and sirohydrochlorin was eluted with 50 mM Tris-HCl buffer, pH 8.0 containing 1 M NaCl. The activity was measured in triplicate using a Shimadzu UV-1203 spectrophotometer. The assays were performed in 1 ml reaction in 50

mM Tris-HCl buffer, pH 8.0 with 100 mM NaCl, with 4.2 μ M of sirohydrochlorin, 20 μ M of Co²⁺ or Fe²⁺ and with different amounts of enzyme.

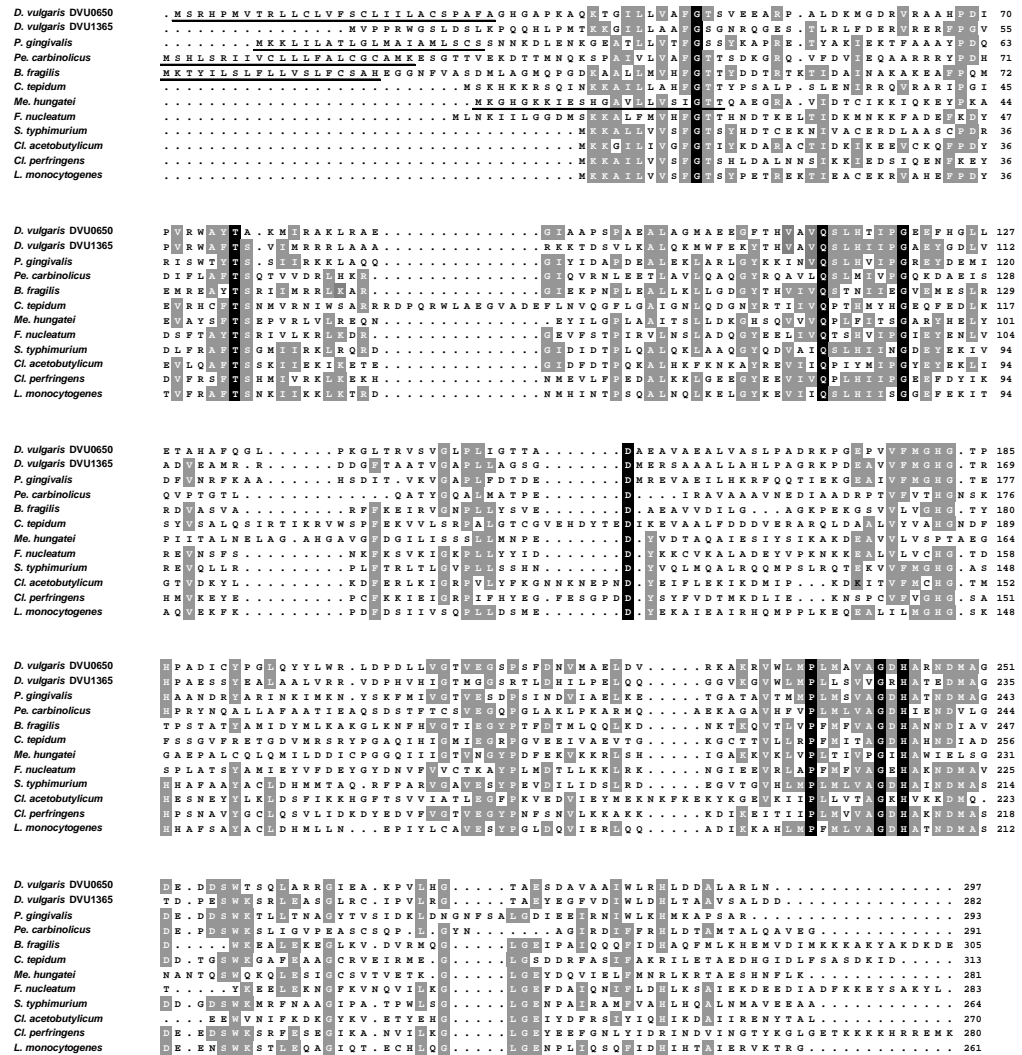
Complementation of *E. coli cysG* mutant strain with *D. vulgaris* cobaltochelataes. The genes from pET-28a(+)DVU0650, pET-28a(+)Δ28DVU0650 and pET-28a(+)DVU1365 were subcloned in pETac, a plasmid under the control of the *tac* promoter. The resulting plasmids, pETac-DVU0650, pETac-Δ28DVU0650 and pETac-DVU1365, were transformed into *E. coli* 302Δa, a strain deleted in the *cysG* and harbouring plasmid pCIQ-*sirCcobA* for expression of the *M. thermoautotrophicus sirC* and *Paracoccus denitrificans cobA* genes. The *E. coli cysG* mutant strain containing the recombinant plasmids was selected on LB plates supplemented with 100 μ g/ml ampicillin and 35 μ g/ml chloramphenicol. Strain *E. coli* 302Δa was also transformed with the plasmid pKK223.2-*cysG*, that expresses the *E. coli* CysG, or with an empty pETac plasmid and grown on MM plates in the absence and in the presence of cysteine, in order to provide the positive and negative controls of the experiment, respectively.

Bioassay for detection of vitamin B₁₂ in *D. vulgaris* cells. *Desulfovibrio vulgaris* Hildenborough was grown anaerobically at 37°C for 48 h in sulfate/lactate growth medium supplemented with a trace element solution (26). The cells were collected and the bioassay was performed according to the technique previously described (27).

4.3 - Results

The analysis of the *D. vulgaris* Hildenborough genome revealed the presence of two gene loci, DVU0650 and DVU1365, encoding putative cobaltochelataes that share ~30 % amino acid sequence identity with CbiK from *S. enterica* and *Porphyrimonas gingivalis* (28, 29) (Figure II-4.1). In

order to study the physiological and biochemical role of these two genes, they were amplified, cloned and the enzymes produced, purified and characterized.



***D. vulgaris* cobaltochelatae DVU0650 contains a haem *b*.** After overproduction in *E. coli* and isolation, the soluble fraction containing the purified DVU0650 exhibited an intense pink-orange colour, which was shown to be due to the presence of a haem cofactor (1 mol/dimer). For convenience, DVU0650 was termed CbiK^P (periplasmic form of CbiK).

As-isolated CbiK^P migrated in SDS-PAGE with an apparent molecular mass of 28 kDa (Figure II-4.2A), that could correspond to a protein in which a cleavage of 28 residues had occurred. In fact, the presence of a signal peptide was confirmed by N-terminal sequencing which showed that the mature protein starts at residue 29. Gel exclusion chromatography of purified CbiK^P suggests that the protein exists in solution as a homotetramer (data not shown).

A UV-visible spectrum of the oxidized form of the protein exhibited a Soret band at 414 nm and a broad band between 515 and 580 nm; upon reduction with sodium dithionite the Soret band shifted to 424 nm with concomitant appearance of two defined bands at 530 nm and at 560 nm, suggesting the presence of a haem *b* prosthetic group (Figure II-4.2B). To elucidate further the type of haem present, pyridine hemochrome analysis was performed in the total protein and in its isolated cofactor. In both cases the redox spectrum presented a β band with an absorption maximum of 555 nm confirming the presence of a haem *b* type (19). To characterize the haem *b* present in DVU0650, a redox titration was performed and a mid-point redox potential of -130 mV was determined (Figure II-4.2C). NMR spectroscopy was used to infer the nature of the haem ligands through the analysis of the reduced form of the protein. The sample presented a spectrum typical of low-spin paramagnetic haem protein and upon reduction the paramagnetic signals in the high frequency region disappeared. The low frequency region of the spectrum did not show the fingerprint for a diamagnetic haem protein with a histidine-methionine coordination (30, 31), *i.e.*, the signal of the methionine ϵ methyl group usually observed at \sim -3 ppm

was absent, suggesting that the haem has a bis-histidinyl coordination (data not shown). EPR analysis of the as-isolated DVU0650 exhibited g -values of 2.94, 2.26 and 1.53, indicative of the presence of a low spin haem group (Figure II-4.2D).

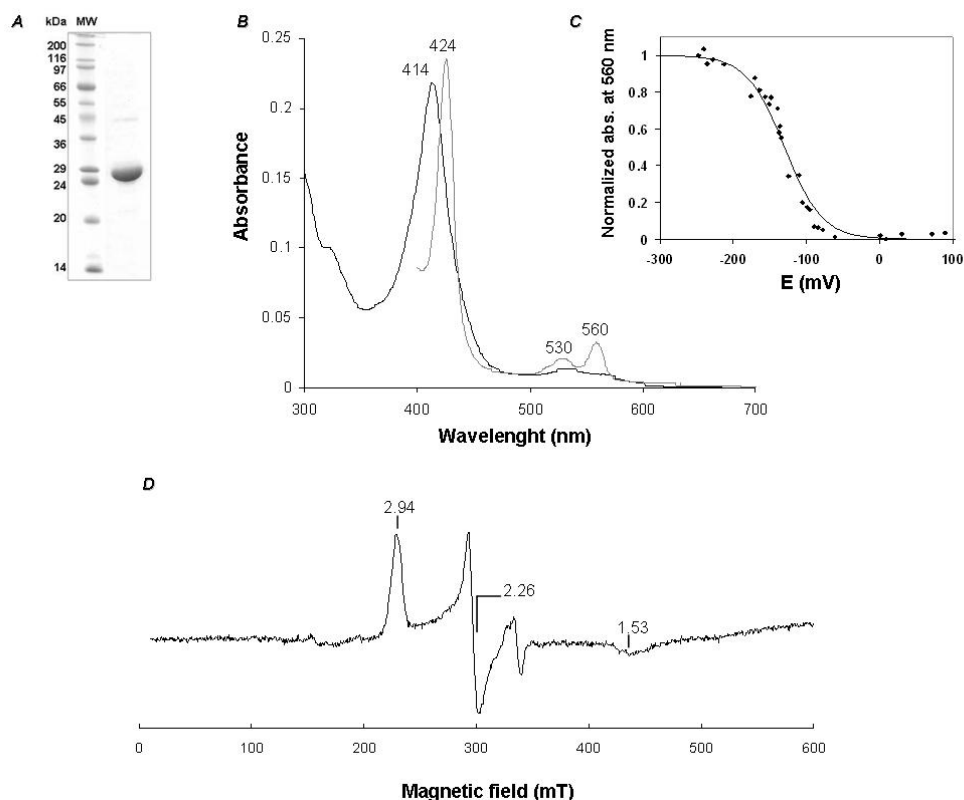


Figure II-4.2 - Biochemical features of the DVU0650 CbiK^P cobaltochelate of *D. vulgaris*. *Panel A*: SDS-PAGE gel of the purified CbiK^P protein and molecular weight marker (MW). *Panel B*: UV-visible spectra of oxidized (black line) and reduced (gray line) CbiK enzyme indicating the presence of a haem *b*. *Panel C*: Redox titration of the haem *b* of *D. vulgaris* CbiK^P with a redox mid-point potential of -130 mV. *Panel D*: EPR spectrum of CbiK^P protein with g values typical for the presence of low spin haem.

CbiK^P (DVU0650) is a sirohydrochlorin cobaltochelate. The DVU0650 enzyme purified under aerobic conditions had activity values very similar to those obtained with the protein purified anaerobically. A truncated protein

that lacks the signal peptide was also produced (see Materials and Methods). This protein, named $\Delta 28\text{CbiK}^{\text{P}}$, exhibited the same molecular mass profile as observed for CbiK^{P} on both SDS-PAGE gel and gel exclusion chromatography (data not shown). However, no haem content was determined in the $\Delta 28\text{CbiK}^{\text{P}}$ protein. The chelatase activity could be then assessed for two forms of the enzyme, one containing the haem cofactor and another lacking the chromophore, allowing us to infer the influence of the haem in the enzymatic activity of CbiK^{P} . The chelatase activity assays revealed that both CbiK^{P} and $\Delta 28\text{CbiK}^{\text{P}}$ were able to insert cobalt and iron into sirohydrochlorin and that the activities were not affected by the presence of the haem, since both proteins had very similar specific activities. These results also showed that the two forms of the enzymes have a slightly higher activity with cobalt than with iron (Table II-4.1).

Table II-4.1- Sirohydrochlorin cobalt and iron chelatase specific activity measured for DVU0650, $\Delta 28\text{DVU0650}$ and DVU1365 enzymes.

Protein	Activity ^a	
	(nmol.min ⁻¹ .mg ⁻¹ of protein)	
	Co ²⁺	Fe ²⁺
DVU0650	22 ± 3	13 ± 3
$\Delta 28\text{DVU0650}$	25 ± 2	18 ± 2
DVU1365	4 ± 0.1	1.5 ± 0.1

^aThe activity values are presented with the standard deviation

DVU1365 is a second sirohydrochlorin cobaltochelatae. As mentioned above, *D. vulgaris* DVU1365 encodes a second putative CbiK cobaltochelatae that shares 39 % amino acid sequence identity with CbiK^{P} (DVU0650) (Figure II-4.1) but lacks an N-terminal transit peptide. Consequently, this form of CbiK was termed CbiK^{C} (for cytosolic). The gene was cloned and the enzyme was overproduced in *E. coli*. In contrast to CbiK^{P} ,

no cofactor was detected in the purified CbiK^C. The purified CbiK^C migrates on a denaturing gel with a molecular mass of approximately 31 kDa. The chelatase activity was assayed with both cobalt and iron in the same conditions described before. CbiK^C has an approximate order of magnitude lower activity than CbiK^P with a specific activity of 4 nmol.min⁻¹.mg⁻¹ with cobalt and 1.5 nmol.min⁻¹.mg⁻¹ with iron (Table II-4.1).

Δ28CbiK^P and CbiK^C complement an *E. coli* sirohydrochlorin ferrochelatase mutant strain. Previously, it has been shown that cobaltochelatases can complement a sirohydrochlorin ferrochelatase deficient strain of *E. coli*. This is due to the lack of metal ion specificity displayed by these chelatases and their ability to insert either ferrous or cobalt ions into sirohydrochlorin (10, 29). In order to investigate whether the *D. vulgaris* CbiK^P and CbiK^C were able to complement the sirohydrochlorin ferrochelatase deficiency of an *E. coli* strain, the genes were cloned into a pETac plasmid and introduced in *E. coli* 302Δa that harboured pCIQsirCcobA (i.e. a strain that is able to make sirohydrochlorin but not sirohaem). To overcome the potential problem of CbiK^P being exported to the periplasm in *E. coli*, the complementation was performed with the truncated version. Both CbiK^C and Δ28CbiK^P cobaltochelatases were found to complement the mutant strain as observed by growth of the transformed strains on minimal medium in the absence of cysteine. Thus both enzymes are able to act as sirohydrochlorin ferrochelatases in the biosynthesis of sirohaem.

***D. vulgaris* produces vitamin B₁₂.** *D. vulgaris* cells were grown on a defined medium in the absence of any exogenous cobalamin. After growth, the cells were harvested and analysed for the level of cobalamin that was produced. By bioassay the strain was found to produce cobalamin to a level of about 10 nmoles per liter of culture.

4.4 - Discussion and conclusion

In this work we characterized two putative CbiK cobaltochelataes present in the genome of *D. vulgaris* encoded in the loci DVU0650 and DVU1365. Production of the recombinant DVU0650 (CbiK^P) revealed that the protein was processed to a form that lacks the first 28 amino acid residues in accordance with the predicted prokaryotic leader sequence. A leader sequence in cobaltochelataes has so far only been reported for the *P. gingivalis* enzyme (28); however, a search in the protein database revealed that other prokaryotic CbiKs are also predicted to contain signal peptide sequences (Figure II-4.1).

The presence of redox centres, whose role remains unclear, has been previously reported in some prokaryotic chelataes. A recent study revealed that a [4Fe-4S]^{+2/+1} centre exists in the CbiX^L cobaltochelataes from *Bacillus megaterium* and *Synechocystis* PCC6803 (6) whereas several bacterial protoporphyrin ferrochelataes contain [2Fe-2S]^{+2/+1} clusters (32, 33). However, the presence of a haem in a chelatase enzyme is so far unique. Our study showed that in *D. vulgaris* CbiK^P cobaltochelatae the haem is not required for the fully enzyme activity since CbiK^P and Δ28CbiK^P were able to insert cobalt and iron into sirohydrochlorin and the absence of the haem did not affect the activity of metal insertion.

Insertion of iron into sirohydrochlorin, yielding sirohaem has been reported for other cobaltochelataes but in a lower ratio compared to cobalt insertion (5, 29). The *D. vulgaris* CbiK^P and CbiK^C cobaltochelataes were also found to have specific activities with iron lower than that measured with cobalt. Nevertheless, the two *D. vulgaris* chelataes were able to complement the *E. coli cysG* mutant strain showing that *in vivo*, they can both insert iron into sirohydrochlorin, thereby rescuing the cell sirohydrochlorin ferrochelatae deficient phenotype.

The presence of two putative genes encoding cobaltochelataes is also found in other genomes, including *D. desulfuricans* G20 (gene locus Dde2181

and Dde3103), *Clostridium acetobutylicum* (gene locus CAC1373 and CAC0583), *Methanospirillum hungatei* (gene locus Mhum0266 and Mhun0668), *Methanobrevibacter smithii* (gene locus Msm1280 and Msn1281) and in the Methanogenic archaeon RC-I (gene locus RCIX2006 and RCIX98). The fact that in *D. vulgaris* one of cobaltochelatases is periplasmic and the other is cytoplasmic suggests that the two chelatases may contribute to different processes.

In *P. gingivalis*, an outer membrane hemin-binding protein, termed IhtB, is proposed to be involved in iron transport and has ~38 % sequence identity with the CbiK from *S. enterica* (34). The *ihb* gene is located in a gene cluster *ihbABCDE*, where the other genes are predicted to encode iron transporters of the ABC type (34). The protein was shown to be an outer membrane hemin-binding protein and it was suggested that the protein may be involved in the removal of iron from haem prior to uptake by *P. gingivalis* (34). The *P. gingivalis* protein was also shown to act as a functional cobaltochelatase in both *in vivo* and *in vitro* studies, although the organism does not appear to have a complete cobalamin biosynthetic pathway (28). Moreover, in *Dichelobacter nodosus* *cbiK* was shown to be under the control of the ferric uptake regulator (Fur) as was the gene for an orthologue of the periplasmic iron binding protein YfeA, itself a component of an ABC transporter system that is involved in iron uptake (35). It is interesting to note that the DVU0650 gene is also located in a putative operon, predicted by the Softberry FGENESB program (36) that contains genes for iron transport proteins (Figure II-4.3). Cumulatively, this information provides a case for *D. vulgaris* CbiK^P being part of an iron or haem acquisition system. The presence of the protein in the periplasm means that it cannot participate in cobalamin biosynthesis as this process takes place in the cytosol. Thus, *D. vulgaris* has two distinct *cbiK* genes, which encode proteins that can both act as cobaltochelatases. The CbiK^C is likely to be the enzyme associated with cobalamin biosynthesis as this protein is produced in the cytoplasm without

any transit peptide. CbiK^P is moved to the periplasm and is likely to be associated with the iron transporter system. The actual role of CbiK^P in iron transport will require further investigation.

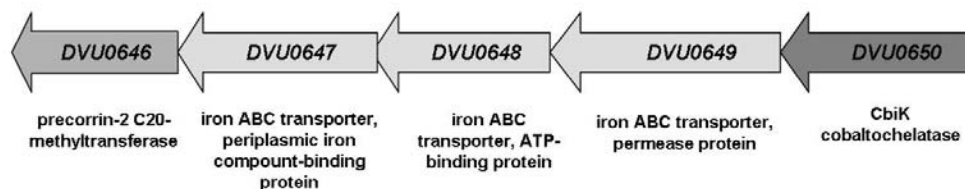


Figure II-4.3 - *D. vulgaris* genomic organisation of the predicted operon encoding DVU0650 CbiK^P cobaltochelatase, DVU0646 CbiL precorrin-2 methyltransferase and three genes for proteins involved in the putative iron ABC transport system (DVU0647, DVU0648 and DVU0649).

Tetrapyrrole biosynthesis and modification in sulfate reducing bacteria is still poorly understood at the biochemical and molecular level. The production of guanylcobamide and hypoxanthylcobamide by *D. vulgaris* was reported a few years ago and the addition of 5,6-dimethylbenzimidazole to *D. vulgaris* culture lead to the production of cyanocobalamin (vitamin B₁₂) (26). Furthermore, analysis of the *D. vulgaris* Hildenborough genome shows the presence of all the genes required for the anaerobic production of vitamin B₁₂ (Table II-4.2), with the exception of precorrin-6x reductase (*cbiJ/cobK*), which within the anaerobic metal-reducing δ -proteobacteria, is only found in the genome of *Desulfuromonas* sp. (37).

Although the requirement of vitamin B₁₂ and/or related corrinoids in *Desulfovibrio* for growth is still unclear, the ability of *D. vulgaris* to produce vitamin B₁₂ was demonstrated in this work.

Table II-4.2- Genes present in the genome of *D. vulgaris* Hildenborough which encode putative enzymes that are required for the anaerobic production of vitamin B₁₂.

Gene locus number	Gene name	Enzyme
DVU1693	<i>gltX-1</i>	gltX-1 glutamyl-tRNA synthetase
DVU1461	<i>hemA</i>	glutamyl-tRNA reductase
DVU3168	<i>hemL</i>	glutamate-1-semialdehyde-2,1-aminomutase
DVU0856	<i>hemB</i>	delta-aminolevulinic acid dehydratase
DVU1890	<i>hemC</i>	porphobilinogen deaminase
DVU0734	<i>cysG-1</i>	uroporphyrinogen III synthase/methyltransferase
DVU1463	<i>cysG-1</i>	sirohaem synthase, N-terminal domain protein
DVU0650	<i>cbiK</i>	cobalt chelatase
DVU1365	<i>cbiK</i>	cobalt chelatase
DVU0646	<i>cbiL/cobI</i>	precorrin-2 methyltransferase CbiI
DVU2750	<i>cbiD</i>	cobalamin biosynthesis protein CbiD
DVU3169	<i>cbiG</i>	cobalamin biosynthesis protein CbiG
DVU3170	<i>cbiH/cobJ</i>	precorrin-3b c17-methyltransferase CobJ
DVU2748	<i>cbiF/cobM</i>	precorrin-4 C11-methyltransferase CobM
DVU2749	<i>cbiET/cobL</i>	precorrin-6y methylase cobL
DVU3087	<i>cbiC/cobH</i>	precorrin-8X methylmutase CobH
DVU3086	<i>cbiA/cobB-2</i>	cobyrrinic acid a,c-diamide synthase CobB-2
DVU0405	<i>cbiA/cobB-1</i>	cobyrrinic acid a,c-diamide synthase CobB-1
DVU1403	<i>cobA/cobO</i>	cob(I)alamin adenosyltransferase CobO
DVU0816	<i>cbiP/cobQ</i>	cobyrric acid synthase CobQ
DVU2237	<i>cbiB/codCD</i>	cobalamin biosynthesis protein CbiB
DVU1007	<i>cobU/cobP</i>	cobinamide kinase/cobinamide phosphate guanylyltransferase CobU
DVU0914	<i>cobS/cobY</i>	cobalamin 5'-phosphate synthase/cobalamin synthase CobS
DVU3279	<i>cobT</i>	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase CobT

4.5 - Acknowledgments

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Crystal structure of a haem-containing cobaltochelatase (CbiK^P) of *Desulfovibrio vulgaris* Hildenborough[†]

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Summary

Sulphate-reducing bacteria of the genus *Desulfovibrio* are relevant microorganisms regarding ecological and biotechnological aspects. Recent studies on the tetrapyrrole biosynthetic pathway operative in these bacteria showed that *D. vulgaris* Hildenborough has two CbiK cobaltochelatases, CbiK^P and CbiK^C, that perform the *in vitro* cobalt and iron chelation into sirohydrochlorin, yielding Co-sirohydrochlorin or sirohaem, respectively. CbiK^P is a tetrameric protein and has a biochemical feature distinct from all the so far known chelatase enzymes, which is the presence of a haem *b* cofactor. To understand how this protein is structurally organised, the X-ray crystal structure of the CbiK^P was determined, in two different states: as-isolated and co-crystallised in the presence of cobalt, at 1.9 Å and 2.4 Å resolution, respectively. The overall monomeric structure of CbiK^P reveals a bilobial topology and that the haem *b* cofactor is located at the interface between two monomers, coordinated by two symmetrically located histidines, namely His96 of each monomer. The structure co-crystallised with cobalt, which constitutes, so far, the first structure of a cobalt loaded cobaltochelatase, revealed that the protein binds cobalt via His154, Glu184 and His216. Comparison of the loaded and unloaded structures revealed that His154 is the only residue that shifts, probably to allow the coordination of the cobalt atom.

‡ The work described in this Chapter is part of a manuscript in preparation:

Susana Lobo and Lígia M. Saraiva were involved in the expression and purification of the CbiK^P protein and in the analysis of the results. Célia Romão, Pedro Matias and Maria Arménia Carrondo performed all the work required for the determination and interpretation of the 3D Structure.

5.1 - Introduction

Modified tetrapyrroles are key elements for life since they serve as cofactors and pigments in several biological processes. Examples are haems, sirohaem, vitamin B₁₂, coenzyme F₄₃₀ and chlorophyll, whose assembly requires insertion of a specific metal ion into the tetrapyrrolic structure, via chelatase enzymes, that differ in size and ATP requirement (1). The protoporphyrin IX magnesium chelatase (BhlH-I-D), involved in chlorophyll synthesis, is a heterotrimer and requires ATP for catalysis (2), while chelatases involved in haem and sirohaem biosynthesis are monomers or homodimers and do not utilize ATP (3, 4). Cobaltochelatases act in the biosynthetic pathway of vitamin B₁₂, being cobalt inserted into the tetrapyrrole ring by the CbiK and CbiX cobaltochelatases, in the anaerobic route, and by hydrogenobyrinic acid *a,c*-diamide cobaltochelatase (CobN-S-T) in the aerobic (oxygen-dependent) pathway (1, 5). CbiX enzymes have two forms, the short form (CbiX^S), present in archaea, and the long form (CbiX^L) found in bacteria (1, 6). The fact that CbiX^L has approximately the double of the size of CbiX^S and that both N- and C-terminal domains share high degree of similarity to CbiX^S, led to the suggestion that this long form results from gene duplication and fusion of *cbiX^S* (1). Although CbiK has a very low amino acid sequence identity with CbiX^S or CbiX^L, all proteins exhibit a similar structure topology (1, 7, 8). Furthermore, CbiK of *Salmonella typhimurium* (8) also shows similar structural features with the protoporphyrin IX ferrochelatase (HemH) of *Bacillus subtilis* (9).

Proteins isolated from *Desulfovibrio* species that belong to the group of sulphate reducing bacteria (SRB), have unusual types of modified tetrapyrroles such as iron-uroporphyrin I, iron-coproporphyrin III and Co^{III}-sirohydrochlorin (10-12). *D. vulgaris* Hildenborough was recently shown to contain two CbiK cobaltochelatases, one cytoplasmic (CbiK^C) and one periplasmic (CbiK^P) that perform cobalt chelation into sirohydrochlorin to yield Co-sirohydrochlorin (or ultimately vitamin B₁₂). Some prokaryotic

chelataes were reported to contain iron-sulphur clusters (*e.g.* the protoporphyrin IX ferrochelatae of *Mycobacterium tuberculosis*, that contains a [2Fe-2S] cluster and the CbiX^L cobaltochelataes from *B. megaterium* and *Synechocystis* PCC6803, that contain a [4Fe-4S] cluster (6, 13)) whose function remains unknown. The study of the CbiK^P of *D. vulgaris* revealed the presence of a haem *b* (14), constituting a unique feature, so far described, in the chelatae family of enzymes. Since the haem of *D. vulgaris* CbiK^P was shown not to be required for sirohydrochlorin cobalt or iron chelatae activity (14), its function remains to be established.

To gain insight into the potential function of *D. vulgaris* CbiK^P, the crystal structure was determined and compared with the bacterial chelatae structures so far available.

5.2 - Materials and Methods

Protein crystallisation and soaking. CbiK^P protein of *D. vulgaris* was overexpressed and purified as previously described (14) and used in the crystallisation trials at a concentration of 10 mg/ml, in buffer 20 mM Tris-HCl pH 7.5. Crystallisation conditions were screened using the nano-drop crystallisation robot (Cartesian, Genomic Solutions) and the Classic Screen from Nextal. Crystals were grown using the hanging drop vapour diffusion method, at 20 °C. The best crystals were obtained by mixing 1.0 µl of the protein with 2.0 µl of the reservoir solution and equilibrating the drop over 0.5 ml of the reservoir, in a solution containing 100 mM Tris-HCl pH 8.5 and 2.0 M ammonium sulphate (Condition #30 of the Classic Screen from Nextal). The crystals were orange coloured due to the presence of the haem *b* (Figure II-5.1). Two crystals were analysed: Crystal A, protein as isolated, crystallised by mixing 1.0 µl CbiK^P (10 mg/ml) with 2.0 µl reservoir solution; Crystal B, protein co-crystallised with cobalt, by mixing 1.0 µl CbiK^P protein (10 mg/ml) with 1.8 µl reservoir solution plus 0.2 µl cobalt chloride (1M).

X-ray data collection and processing.

The crystals were cryo-protected with mother liquor solution supplemented with 20 % glycerol. The crystals belong to the space group I422 with a Matthews coefficient (V_m) of $3.88 \text{ \AA}^3 \text{ Da}^{-1}$ and $3.98 \text{ \AA}^3 \text{ Da}^{-1}$, corresponding to an estimated solvent content of 68 % and 69 % for crystal A and crystal B, respectively. One monomer is present per asymmetric unit.

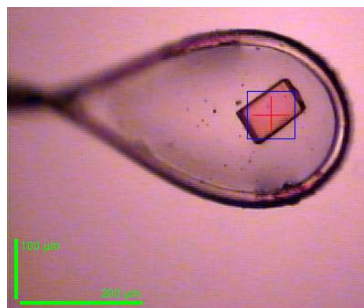


Figure II-5.1 - Crystal in a loop in cryo-solution, freezed in liquid nitrogen just before the data collection.

X-ray diffraction data were collected at the European Synchrotron Radiation Facility, using the beamlines ID14-1, for crystal A and BM14, for crystal B, to 1.9 \AA and 2.4 \AA , respectively.

The dataset from crystals A and B was integrated using MOSFLM (15) and XDS (16), respectively. Scaling, merging and conversion to structure factors was performed with SCALA and TRUNCATE (17) for both datasets. The statistics of each dataset are presented in Table II-5.1.

Phasing and refinement of the structure. The structure was determined by MAD method using the four datasets from crystal B. Two of the energies (peak and inflection point) were calculated with the CHOOCH program (18) from an X-ray fluorescence spectrum of crystal B, taken near the K-absorption edge of cobalt. Two additional energies (low-energy remote and high-energy remote) were chosen (Table II-5.1).

Using the HKL2MAP (19) graphical user interface, the 4-wavelength MAD dataset from crystal B was scaled, and analysed with SHELXC (20). The Co^{2+} heavy atom sub-structure was determined with SHELXD (21-23) and the phase problem solved with SHELXE (24). The best solution from SHELXD was with 50 trials that gave two major Co^{2+} . The SHELXE

calculations provided a clear discrimination between the correct and the inverted sub-structure solutions. The solution obtained had a mean figure of merit (FOM) of 56.4 %.

The ARP/wARP program (25) was used for model building. The final model built 251 amino acid residues out of 269, with an R -factor of 19 % and an R_{free} of 24.2 %.

Table II-5.1 - Data collection statistics.

	Structure A As isolated	Structure B Cobalt co-cristallisation			
ESRF Beamline	ID14 EH1	BM14	BM14	BM14	BM14
Space group	I 4 2 2	I 4 2 2	I 4 2 2	I 4 2 2	I 4 2 2
Wavelength (Å)	0.934	1.60458 pk Co	1.60556 ip Co	1.61019	1.42363
Unit cell (Å)	121.61, 121.61 120.28 90,90, 90	121.41, 121.41 121.07 90,90, 90	121.42, 121.42 121.02 90,90, 90	121.40, 121.40 120.40 90,90, 90	121.42, 121.42 120.97 90,90, 90
Resolution (Å)	49.57-1.90 (2.00-1.90)	49.45-2.4 (2.53-2.40)	49.45-2.4 (2.53-2.40)	49.45-2.4 (2.53-2.40)	49.45-2.4 (2.53-2.40)
R_{merge}	0.166 (0.435)	0.092 (0.583)	0.082 (0.502)	0.093 (0.620)	0.067 (0.411)
R_{pim}	0.067 (0.170)	0.035 (0.225)	0.031 (0.193)	0.050 (0.348)	0.036 (0.227)
I/σ	2.6 (1.6)	7.8 (1.3)	8.7 (1.6)	7.9 (1.3)	10.9 (1.9)
Wilson B-factor (Å ²)	22.72	38.18	37.04	35.47	36.21
Completeness (%)	99.9 (100.0)	99.8 (98.5)	99.8 (98.6)	98.0 (98.8)	97.5 (96.6)
Multiplicity	7.2 (7.4)	14.9 (14.6)	14.6 (14.6)	7.6 (7.4)	7.7 (7.6)
Number observations	257339 (37699)	268739 (37274)	268613 (37210)	133606(18422)	134448 (18669)
Number unique reflections	35692(5122)	18035(2546)	18046(2559)	17558(2491)	17470(2469)

ESRF: European Synchrotron Radiation Facility; Values under brackets are for the highest resolution shell.

$R_{merge} = (\sum(I - \langle I \rangle) / \sum(I))$; where I is the intensity measured for a given reflection, $\langle I \rangle$ is the average intensity for multiple measurements of this reflection.

$R_{pim} = \sum [1/(N-1)]^{1/2} (\sum(I - \langle I \rangle) / \sum(I))$; where I is the intensity measured for a given reflection, $\langle I \rangle$ is the average intensity for multiple measurements of this reflection.

Structure A was solved by molecular replacement using PHASER (26) and the model obtained for Structure B. Refinement of both Structures was performed using cycles of REFMAC (27) and COOT (28). Water molecules were placed automatically with REFMAC and ARP/wARP (17, 27). The refinement statistics of each Structure are presented in Table II-5.2. The Ramachandran plot shows no residues in the disallowed regions for both Structures. All the stereochemical criteria, were calculated by SFCHECK (17, 29), and satisfy or are better than the average values of Structures

determined at a similar resolution. The overall G-factor is 0.1 in both Structures A and B.

Table II-5.2 - Refinement statistics for CbiK^P Structures A and B.

	Structure A 1.9 Å	Structure B 2.4 Å
R (%) (no. of reflections)	18.4 (33904)	18.0 (17055)
R _{free} (%) (no. of reflections)	21.1 (2458)	22.9 (1205)
R.m.s. deviations from standard geometry (Å):		
<i>bond lengths</i>	0.018	0.017
<i>bond angles</i>	1.465	2.021
<i>bond torsions</i>	5.211	9.009
Number non-hydrogen atoms:		
ALL	2336	2193
Protein	2022	2009
Water	223	111
Haem <i>b</i>	1	1
Co	0	1
Sulphate	3	3
Glycerol	3	1

Sequence alignment. The selected amino acid sequences were aligned using Clustal X (30). Selected protein structures were individually aligned with the Structure A of *D. vulgaris* CbiK^P, using the program Superpose and the Secondary-structure matching (SSM) (31).

5.3 - Results

General structure of CbiK^P. The three-dimensional structure of *D. vulgaris* Hildenborough CbiK^P was solved by MAD method on the cobalt-Kedge using the Crystal B. There is only one subunit on the asymmetric unit but due to the crystal packing, a tetramer is formed, consistent with the previous biochemical studies which showed that this protein is a tetramer in solution (14).

The CbiK^P monomer has a α/β topology, consisting of two domains: domain A (residues 6-117) and domain B (residues 125-269), which are connected by a loop (residues 118-124). The N- and C-terminal of the protein are located close to each other, as shown in Figure II-5.2.

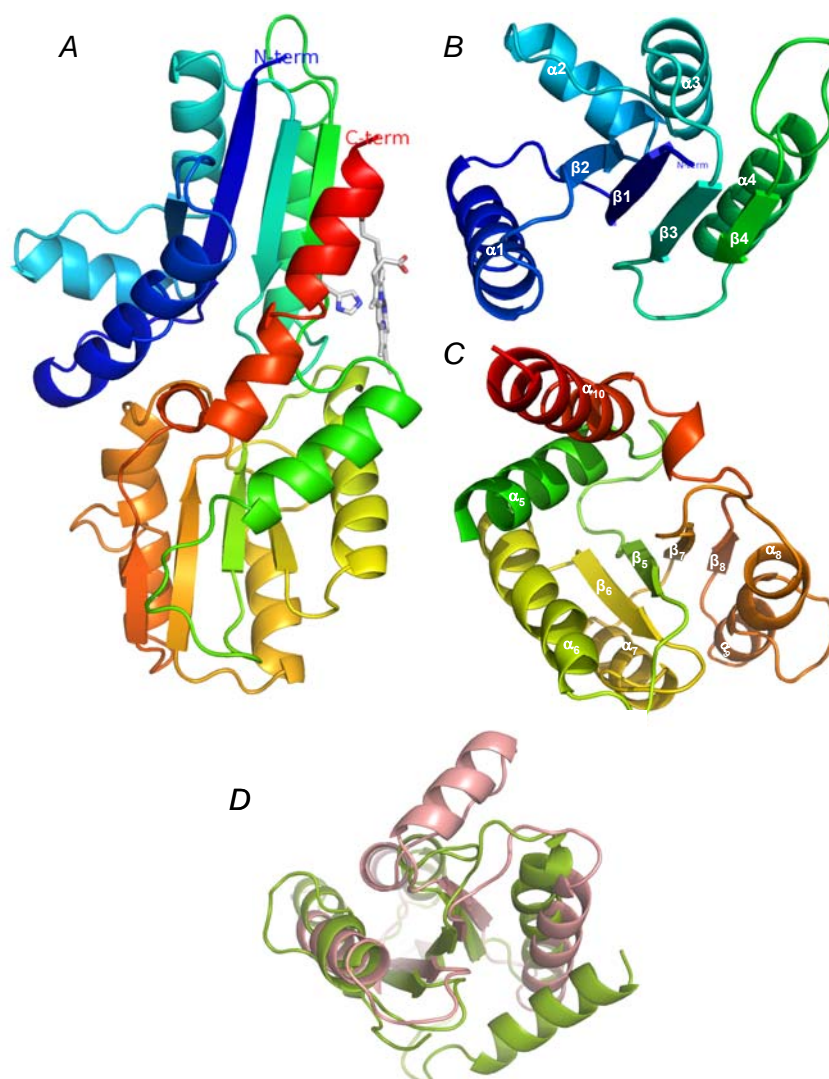


Figure II-5.2 - *A*- *D.vulgaris* CbiK^P monomer overall structure, rainbow coloured from blue (N-terminal) to red (C-terminal). The haem and the amino acid ligand His96 are represented. *B*- Domain A, coloured as in *A*. *C*- Domain B, coloured as in *A*. *D*- Superposition of domains A and B, coloured as light pink and green, respectively.

Two different structures were obtained: Structure A, corresponding to the protein as-isolated (CbiK^P), and Structure B, for the protein co-crystallised in the presence of cobalt (CbiK^P(Co)). For both structures, the

overall fold is essentially the same, with a root mean square (rms) deviation of 0.27 Å. The average B- value for the protein chain is 21.8 Å² and 25.5 Å², for the Structures A and B, respectively. In the two structures, the region between amino acid residue 17 and 30 has a high average B-value of 42.3 Å² for Structure A, and of 65.3 Å² for Structure B. This fact is quite interesting, since this region corresponds to the loop at the end of the β 1-sheet and at the beginning of helix σ 1 (two turns), and which is located near the cleft where the porphyrin putatively binds, as judged by structural homology to other chelatases studied (32). Hence, the flexibility of that this part of the molecule may be related to the need to accommodate the porphyrin molecule.

Domain A is composed by four parallel β -sheets and four-helices namely, α 1, α 2, α 3 and α 4 (Figure II-5.2B). The β -sheets are organised as β 2 - β 1- β 3- β 4, with a slight angle of torsion of *ca.* 65°, and the four α helices surround the core of β -sheets (Figure III-5.2B). The haem *b*, located in between monomers, is coordinated by two symmetric related histidines, His96, located in domain A (Figure II-5.2A).

Domain B is formed by four β -sheets, organised similarly as those of domain A (β 6- β 5- β 7- β 8), is surrounded by fives helices, α 5, α 6, α 7, α -8 and α 9 (Figure II-5.2C), and contains the ligands which coordinate the cobalt, namely residues His154, Glu184 and His216. Helix α 10 of the C-terminal (represented in red on Figure II-5.2A) is connecting the two domains and closely located to helix α 1 of domain A.

To clarify if the two domains of CbiK^P of *D. vulgaris* are structurally similar, the superimposition of both domains was analysed. The secondary structures of the two domains of CbiK^P are superimposed on the four β -sheets and four-helices, with an rms deviation of 3 Å (Figure II-5.2D), with the exception of the helix α 10 (residues 247-266), helix α 2 (in domain A) and helix α 5 (in domain B), that do not superpose any helix in the other domain. Nevertheless, both A and B domains share similarity regarding the secondary structure rearrangement. Furthermore, there are no His residues

in domain A that superimpose the His154 and His216 of domain B suggesting that the catalytic activity is only associated with domain B.

Tetramerisation of CbiK^P. An analysis of the tetrameric CbiK^P (Figure II-5.3A) interfaces, surfaces and assemblies, performed by the PISA server (33), shows that the surface area is 39970 Å² and 41250 Å² and the buried area is 11279 Å² and 10740 Å² for Structures A and B, respectively. Furthermore, the results indicate that the tetramer (in both Structures A and B) is the most energetic favourable assemble, since it exhibits a lower ΔG^{int} . The tetramer may be stabilized by two types of interactions, namely i) the interactions between the vertical monomers, which occur mainly in the haem region since this cofactor is located in between two monomers, ii) the interactions between horizontal monomers, by salt bridges formed between amino acid residues located in different monomers, namely Glu69...Arg54, Glu69...Arg58 and Glu76...Arg58. The two haems *b* are nearly collinear, with the propionate groups pointing towards the centre of the tetramer (Figure II-5.3) and the four monomers of the tetramer are organised in such a way that the clefts for the binding of the porphyrin are faced outwards, being easily accessible to the solvent.

Haem *b* of CbiK^P. The presence of a haem *b* (14), confers to the CbiK^P protein solution and crystal form, an orange-reddish colour. As previously mentioned in *D. vulgaris* CbiK^P, the haem *b* is in between two monomers, thus explaining the value of 0.5 haem/monomer determined in the previous biochemical studies (14). The haem is coordinated by the His96, of each monomer (Figure II-5.3B, II-5.4) with a coordination distance of 2.16 Å to the iron atom. The haem is located in a hydrophobic pocket composed by amino acid residues His158 and His96, Pro159, Pro91 and Phe95 and presents a slightly distorted conformation. The two haems *b* are quite distant to each other with a distance between the two iron atoms of *ca.* 32 Å (Figure II-5.3A).

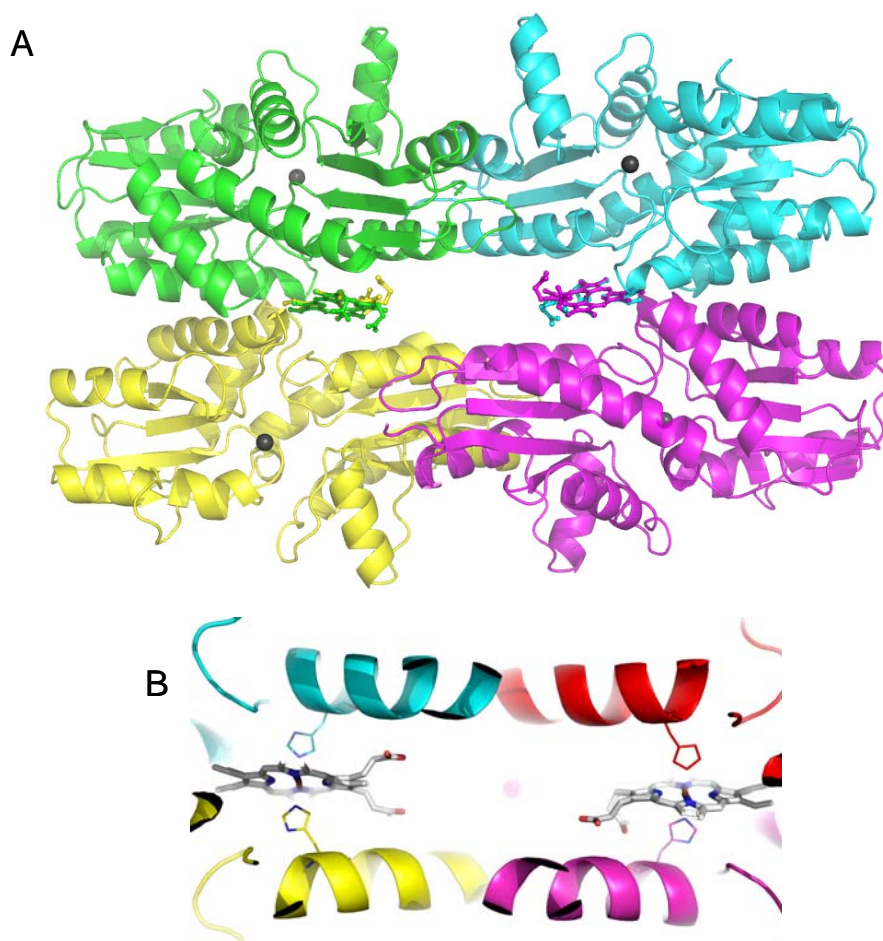


Figure II-5.3- A - CbiK^P tetrameric assembly. Each monomer is coloured differently and the haems in between subunits are represented; the gray spheres in each monomer represent the cobalt sites. B - Closer view on the two haems *b* of CbiK^P.

Two different conformations of the haem are found, in relation to the monomer, with occupancy of 50 %. The core of the porphyrin ring is well superimposed in the two conformations, but the haem propionates and vinyl chains are related by an angle of 90° in the two conformations. This occurrence may be explained by the lack of interactions between the propionates and the amino acid residues of the protein. Only one propionate (ring D) OD2 is H-bond with the ND1 from His103 with a distance of 2.58 Å.

This interaction is only observed in Structure A of CbiK^P, being absent in the CbiK^P(Co) structure (Structure B).

Although the presence of iron-sulphur clusters in chelatases has been observed, as in the human protoporphyrin IX ferrochelatase, which contains an [2Fe-2S] cluster located in a C-terminal extension (34), in the CbiK of *Deinococcus radiodurans*, that contains a [4Fe-4S] located in an extra domain (35), and in the CbiX^L from both *Bacillus megaterium* and *Synechocystis* PCC 6803 that contains a [4Fe-4S] cluster (6), the presence of a haem group, as in *D. vulgaris* CbiK^P, was not yet reported.

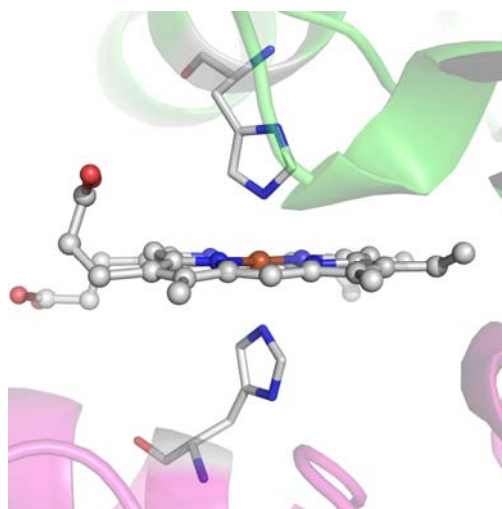
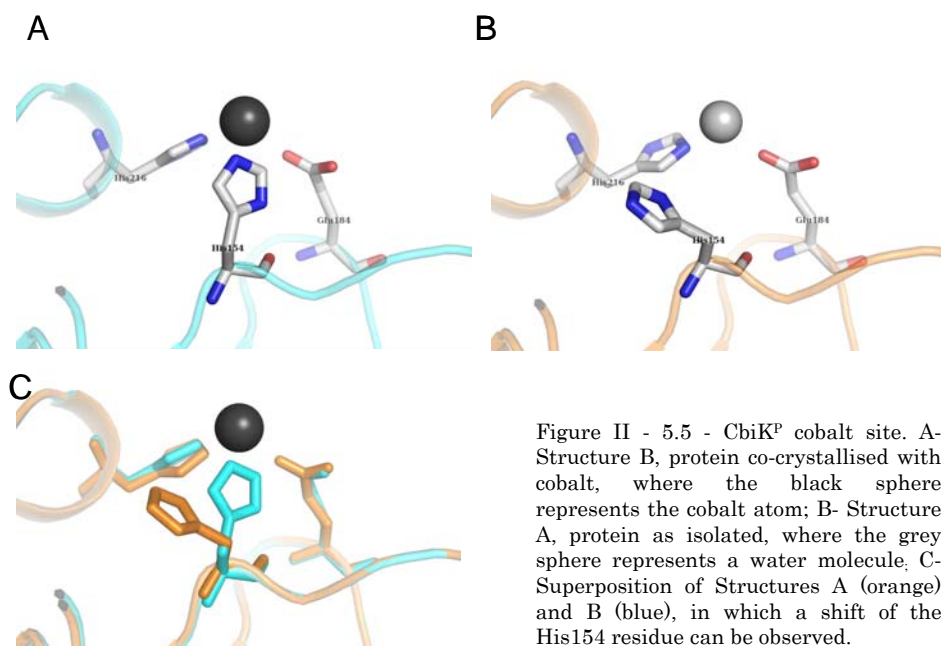


Figure II-5.4- Haem binding site. The iron-haem is coordinated by a bis-His type coordination.

Cobalt binding. Analysis of CbiK^P(Co) structure (Structure B) revealed that the protein binds cobalt via residues His154 (distance Co:N^{ε2} His – 2.37 Å), Glu184 (distance Co:O^{ε2} Glu – 2.95 Å and O^{ε1} Glu – 4.26 Å) and His216 (distance Co:N^{ε2} His – 2.37 Å) (Figure II-5.5A). Structures A and B exhibit differences in this region of the protein. In Structure A, ligand His154 is related 63° in relation to its position in Structure B, implying that in Structure A, this residue is no longer close to the cobalt binding site (Figure II-5.5B,C). Also, in Structure A that lacks the cobalt atom, a water

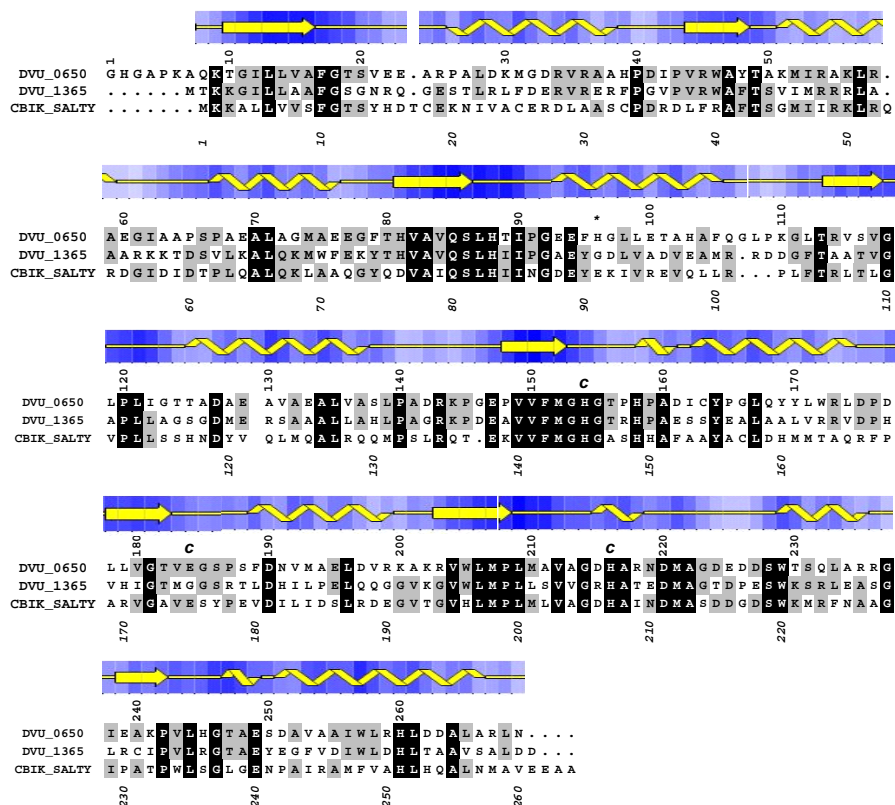
molecule is present (Figure II-5.5B) and the distances of the ligands to the water molecule are: N^{ε2} His154 – 4.43 Å, O^{ε2} Glu184 – 2.64 Å and O^{ε1} Glu184 – 4.33 Å, N^{ε2} His – 2.36 Å. A second water molecule is located at a close distance (2.51 Å) of the first water molecule, but far apart (>3 Å) from any of the ligand residues.



5.4 - Discussion and conclusion

D. vulgaris CbiK^P shares the highest degree of amino acid sequence identity with CbiK^C (43 %). However, the haem *b* ligand, His96, identified in structure of CbiK^P is absent in the sequence of CbiK^C (Figure II-5.6), and accordingly, no haem cofactor was detected in the recombinant CbiK^C protein (14). The second protein with the highest sequence identity is the CbiK of *S. typhimurium* (34 %) that also lacks the His96 ligand (Figure II-5.6).

Figure II- 5.6 - Amino acid sequence alignment of *D. vulgaris* CbiK^P (Dvu0650) with *D. vulgaris* CbiK^C (Dvu1365) and CbiK of *Salmonella typhimurium* (CbiK SALTY). The numbering presented in the top and in the bottom of the alignment, refers to the residues in *D. vulgaris* CbiK^P and *S. typhimurium* CbiK amino acid sequences, respectively. *D. vulgaris* CbiK^P secondary structure is presented in yellow. The symbols *C* and * represent the *D. vulgaris* CbiK^P amino acid residues involved in cobalt and haem binding, respectively. The strictly conserved residues are within black boxes and the conserved residues within dark grey boxes.



The structural alignment with *D. vulgaris* CbiK^P, *S. typhimurium* CbiK (8) and chelatase proteins whose structures are available, namely protoporphyrin IX ferrochelatase (HemH) of *Bacillus subtilis* (9), HemH of *Saccharomyces cerevisiae* (36) HemH of *Human* (34), CbiK of *De. radiodurans* (35) and CbiX^S cobaltochelatase of *Archaeoglobus fulgidus* (7), exhibits an rms deviation between 1.6 and 3 Å (Table II-5.2 and Figure-5.7). The proteins have the same overall structure and α/β topology, and are organised in two core domains. The most similar protein is the CbiK of *S. typhimurium*, with a structural rms deviation of 1.6 Å in a total of 254

residues. Comparison of the histidine haem ligand residue His96 with the corresponding residue in all analysed chelatases show that *D. vulgaris* CbiK^P is the only protein that contains this residue (Table II-5.2).

Table II-5.2 - Data from the structural alignment between *D. vulgaris* CbiK^P monomer and the chelatases of *Salmonella typhimurium* (CbiK), *Bacillus subtilis* (HemH), *Saccharomyces cerevisiae* (HemH), *Human* (HemH), *Archaeoglobus fulgidus* (CbiXS, homodimer A and B) and *Deinococcus radiodurans* (CbiK). The structures were selected by the DALI server (37) and the structure alignment is presented in Figure II-5.7. The rms deviation and the number of amino acid residues that were aligned (Residues) (calculated by SSM) are presented for each alignment. A comparison of the residues involved in the haem binding (His96) and cobalt binding (His154, Glu184 and His216) in *D. vulgaris* CbiK^P, with the equivalent amino acid residues of the chelatases from other organisms is also presented.

Organism	<i>D. vulgaris</i>	<i>S. typhim</i>	<i>B. subtilis</i>	<i>S. cerev</i>	<i>Human</i>	<i>A. fulgidus</i>	<i>De. radiod</i>
PDB code		1qgo	1ak1	1lbq	1hrk	2dj5	2jh3
Z-score		33.5	19.6	17.2	17.3	9.8	13.9
rms (Å)	--	1.6	2.8	3.0	3.0	3.0	3.4
Residues	--	254	223	219	228	212	210
Haem ligand	His96	Glu91	Gln123	Gly172	Gly200	Thr96 ^A	Glu79
Co ligand	His154	His145	His183	His235	His263	His10 ^B	Ala148
Co ligand	Glu184	Glu175	Trp230	Gln276	Lys304	Arg44 ^B	Ser177
Co ligand	His216	His207	Glu264	Glu314	Glu343	His74 ^B	Ala218

Despite the fact that no structure with cobalt was obtained for *S. typhimurium* CbiK, three amino acid residues were proposed to be involved in cobalt binding, namely His145, Glu175 and His207. Furthermore, residue His145 was suggested to affect the degree of selectivity for cobalt, since a *S. typhimurium* His145Ala CbiK mutant was unable to recover the wild type phenotype of an *E. coli* strain deficient in sirohhydrochlorin ferrochelatase activity (8). The structure of the incubated cobalt form of the *D. vulgaris* CbiK^P (Structure B) confirmed this prediction since the corresponding amino acid residues in *D. vulgaris* CbiK^P (His154, Glu184 and His216) are indeed binding the cobalt atom. Furthermore, the comparison of Structures A and B showed that His154 is the only amino acid residue that shifts to allow coordination of the cobalt, which may explain the deleterious effect of His145Ala CbiK mutation of *S. typhimurium*. Comparison of the three amino acid residues that bind cobalt in *D. vulgaris* CbiK^P, with the corresponding

residues in the HemH ferrochelatase enzymes of *B. subtilis*, *S. cerevisiae* and *Human*, CbiK of *De. Radiodurans* and CbiX^S of *A. fulgidus* also show that His154 is the only conserved residue, except in the *De. radiodurans* CbiK protein where an alanine residue replaces the histidine (Table II-5.2).

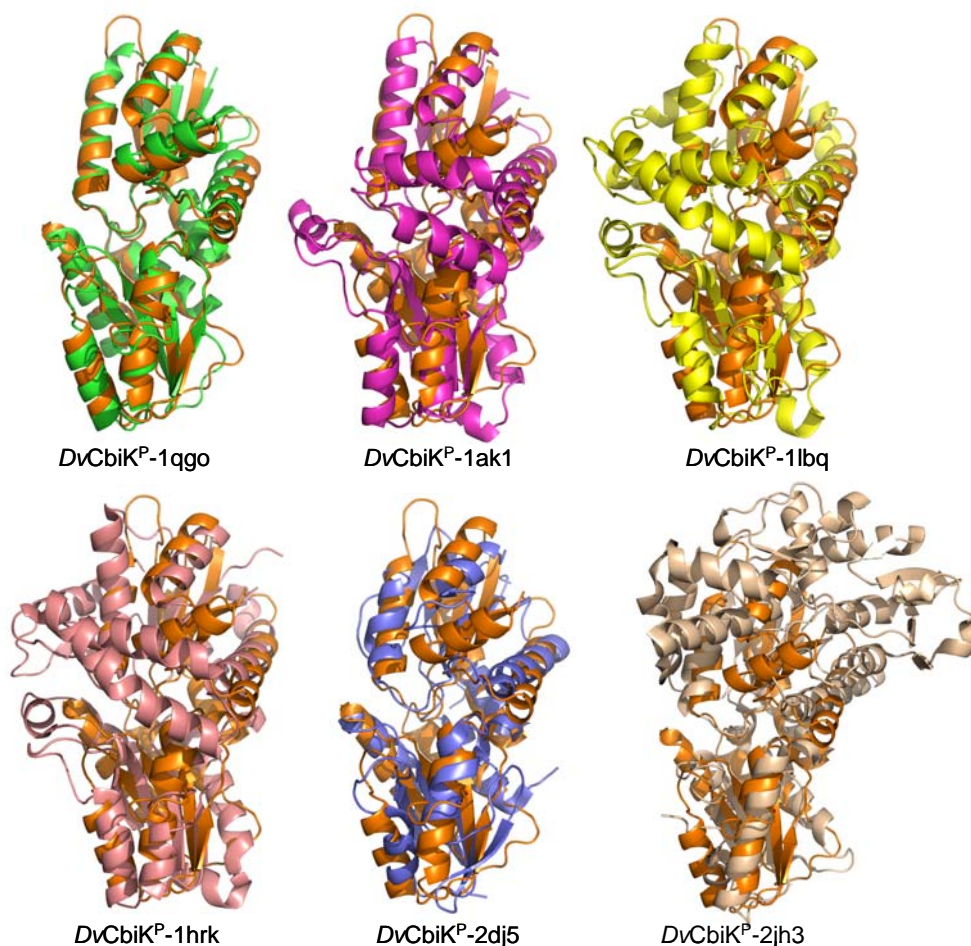


Figure II-5.7 - Structure superposition of the *D. vulgaris* CbiK^P monomer (orange) with the structures of chelatases from different organisms (see page 168 for references). Green - *Salmonella typhimurium* CbiK (1qgo), pink - *Bacillus subtilis* HemH (1ak1), yellow - *Saccharomyces cerevisiae* HemH (1lbq), light pink - *Human* HemH (1hrk), blue - *Archaeoglobus fulgidus* CbiX^S (2dj5) and brown - *Deinococcus radiodurans* CbiK (2jh3).

In summary, in the present work we determined the structure of the only so far, known haem-containing cobaltochelatase, *D. vulgaris* CbiK^P. The protein was successfully co-crystallised with cobalt and the motion of the conserved amino acid residue His154 that could be observed comparing the two determined structures, support the catalytic role of this residue. In the overall *D. vulgaris* CbiK^P tetrameric structure, the four porphyrin binding clefts are facing the outside, which makes this pocket easily accessible for the porphyrin moiety, a fact that may be important for a possible allosteric mechanism/regulation. Although the role of the haem cofactor in *D. vulgaris* CbiK^P remains unknown, it is interesting to note that the calculated ΔG^{int} value of the two determined structures, considering the protein chain plus the haem are -115 and -127.9 kcal/mol for Structures A and B, respectively. The high values of ΔG^{int} value, measured for the protein chain lacking haem, -33 and -39.9 kcal/mol, for Structures A and B, respectively, suggest that the haem confers stability to the tetramer. Interestingly, the iron-sulphur cluster of the *Human* HemH, which is located in a C-terminal extension involved in hydrogen bonding between monomers at the dimer interface, was proposed to be indirectly involved in the dimerization of HemH (34). Similarly, in the *D. vulgaris* CbiK^P, the haem *b* is connecting two monomers and work is under progress to elucidate if the haem is only important for the tetrameric assembling and/or performs other functions.

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Part III

Discussion



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1 - *Desulfovibrio desulfuricans* ATCC 27774 grows in the presence of oxygen

Desulfovibrio species have been shown to perform sulphate reduction in oxygenated environments and to exhibit, to some degree, aerotolerance. The finding of several enzymes that protect against harmful effects of oxygen and the possibility that, at low concentrations, oxygen may serve as electron acceptor to allow energy conservation, provided insights on the way *Desulfovibrio* species deal with oxygen (1, 2). Nevertheless, no significant growth was observed under high oxygen levels and many questions regarding the survival in oxic conditions remain to be elucidated. In this thesis, it was shown that a *Desulfovibrio* species (*D. desulfuricans* ATCC 27774) sustains growth at high oxygen concentrations and a membrane-bound oxygen reductase-like enzyme, with features different from the canonical enzymes, was discovered in *D. vulgaris* Hildenborough.

The studies of *D. desulfuricans* ATCC 27774 under oxic conditions provided the first evidence for the growth of a *Desulfovibrio* organism in an atmosphere containing oxygen in a concentration as high as 18 % (~170 μ M) (Part II/Chapter 1). In contrast to other *Desulfovibrio* sp. (3, 4), we observed that *D. desulfuricans* ATCC 27774 cells do not form aggregates and do not suffer any morphological changes when exposed to oxygen. Under an oxygen atmosphere up to 18 %, the growth curves of *D. desulfuricans* ATCC 27774 showed no lag phase, indicating that this microorganism can rapidly adapt to the presence of oxygen. When submitted to atmospheric oxygen concentrations (21 %, ~200 μ M O₂), *D. desulfuricans* ATCC 27774 cells stopped growing, but no loss in viability was detected. Although no viability loss is also observed when *D. gigas* cells are exposed to 120 μ M oxygen for 8 h (5), *D. aerotolerans* is exposed for 13 h to atmospheric oxygen (4) and *D. vulgaris* Hildenborough is exposed 1 h to ~200 μ M oxygen (6), these cells are not able to grow and experiments using an oxygen concentration of ~880 μ M oxygen, that mimic the oxidative conditions found in cyanobacterial mats (~1 mM of oxygen) and cause severe

oxidative stress, show that the viability of *D. vulgaris* Hildenborough cells decreases significantly (7, 8).

Since *D. desulfuricans* ATCC 27774 respire nitrate, the growth experiments were performed in a nitrate/lactate medium that avoids the chemical reactions between oxygen and reduced sulphur compounds which cause an increase of the detrimental effect of oxygen (Part II/Chapter 1). Under these conditions, the enzymes involved in nitrate respiration pathway, namely nitrate and nitrite reductases, are active. Interestingly, we observed that the enzyme activity of the two proteins was not altered under the oxic conditions used, suggesting that *D. desulfuricans* ATCC 27774 maintains nitrate and nitrite reducing ability under oxygen exposure. Nevertheless, experiments to verify if nitrate was consumed simultaneously with oxygen reduction remained inconclusive (unpublished results). Although reduction of oxygen coupled to lactate oxidation was previously reported in cell extracts of *D. desulfuricans* ATCC 27774, the existence of cell growth in the presence of oxygen was not tested (9, 10). Hence, *D. desulfuricans* ATCC 27774 constitutes, so far, the first example of a sulphate reducing bacteria that may utilize oxygen for growth.

As described in the Introduction of this dissertation (Part I/Chapter 2), *Desulfovibrio* contain several oxygen detoxification systems. In *D. gigas*, superoxide dismutase (Sod) is constitutive, and the catalase activity is enhanced after exposure to 120 μM oxygen (5). In *D. vulgaris* Hildenborough the induction of the *sod* gene depends on the oxygen concentration to which the cells are exposed; the *sod* transcription is not altered in cells exposed to $\sim 0.22 \mu\text{M}$ oxygen and in the presence of $\sim 200 \mu\text{M}$ oxygen *sod* is significantly induced. While the genes encoding catalase and peroxidases are induced in cells submitted to $\sim 0.22 \mu\text{M}$ oxygen, only a weak repression of the peroxidase gene is observed and the transcription of the catalase gene is not significantly altered in cells grown with $\sim 200 \mu\text{M}$ oxygen (11). For *D. desulfuricans* ATCC 27774, superoxide dismutase and

catalase activities were found to increase in response to $\sim 170 \mu\text{M}$ of oxygen, while peroxidase activity was not altered (Part II/Chapter 1). Since in *D. desulfuricans* ATCC 27774 two proteins with superoxide dismutase activity are present (Dfx and Cu-Zn Sod), the superoxide dismutase activity cannot be directly attributed to one of these proteins.

2 - A new type of haem-copper oxygen reductase

The biochemical characterization of the haem-copper oxygen reductase *ccaa3* (Part II/Chapter 2) of *D. vulgaris* Hildenborough was performed in a recombinant truncated form of the enzyme subunit II, consisting solely of the C-terminal domain, allowing us to study the cytochrome *c* domain of this subunit, which was purified as a soluble protein. The spectroscopic data confirmed the predicted binding of two haems *c*, with a 1:1 ratio and with a His-Met haem-iron axial coordination. The reduction potentials of the haems *c* were measured as being -100 mV for the low-potential haem, and +110 mV for the high-potential haem. Since these values were evaluated in a truncated form of subunit II, they may not reflect the ones found in the intact membrane protein.

D. vulgaris Hildenborough *ccaa3* constitutes the first example of a haem-copper oxygen reductase containing two *c*-type haems in subunit II (Figure III-1). Amino acid sequence analysis reveals that the catalytic subunit (subunit I) contains the conserved ligands of the copper centre CuB (12) and all the amino acid residues that form the D- and K-channels, with the exception of the glutamyl residue (Glu278, *Paraccocus denitrificans* numbering) at the end of the D-channel, which, in *D. vulgaris* is replaced by a tyrosine and serine (YS) motif (12). Accordingly, the enzyme of *D. vulgaris* Hildenborough was classified as a Type A2 haem-copper oxygen reductase (12). In *D. vulgaris ccaa3*, the ligands of the binuclear copper centre (CuA) and of the haems *a*- and *a3*- types are conserved (12) and the

two haems *c* binding motifs (CxxCH) are located in the C-terminal domain of subunit II (Figure III-1).

All *Desulfovibrios* contain dihaem haem-copper oxygen reductase enzymes and the comprehensive amino acid sequence comparison of subunit II with ortholog proteins (Part II/Chapter 2), showed a high degree of identity between the two cytochrome *c* domains of the *ccaa₃* enzymes and the single cytochrome *c* domain of the *caa₃* family of proteins. Hence, we proposed that *ccaa₃* enzymes evolved from the *caa₃* oxygen reductases, by gene duplication.

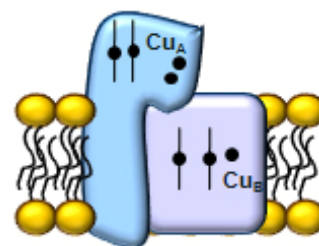


Figure III-1 - Schematic representation of the *ccaa₃* haem-copper oxygen reductase.

Our studies show that the *ccaa₃* haem-copper oxygen oxygen reductase of *D. vulgaris* Hildenborough is a constitutive enzyme. Furthermore, membranes of *D. vulgaris* reduce oxygen in the presence of the cytochrome *c₅₅₃*, revealing the first possible electron donor to the *ccaa₃* enzyme (Part II/Chapter 2). When using an artificial electron donor to oxygen reductases, *D. desulfuricans* ATCC 27774 membranes were also shown to consume oxygen (Part II/Chapter 1). We observed that membranes of *D. desulfuricans* ATCC 27777 contain, at least, two haems of the *b* and *c*-types. However, haem *a*, usually associated with the haem-cooper oxygen reductases of the A and B families, was not detected, a result that raises several hypothesis: i) haem *a* is absent from the membranes, ii) haem *a* is present in the membranes, but its amount is below detection or iii) *D. desulfuricans* ATCC 27777 contains a different type of haem *a*, as proposed for *D. gigas* (13). In the recently sequenced genome of *D. desulfuricans* ATCC 27774 only a deduced gene sequence sharing significantly amino acid identity to a *bd*-type reductase is identified. Hence, the absence of a haem-copper oxygen reductase in *D.*

desulfuricans ATCC 27774 genome, that only became available after conclusion of this work, is consistent with the fact that we could not detect haem *a* in the membranes of this organism. Although the addition of cyanide, a powerful inhibitor of the haem-copper oxygen reductases, caused inhibition of oxygen reduction in the membranes of *D. desulfuricans* ATCC 27774, suggesting the co-existence of a *bd*-type and a haem-copper oxygen reductase in *D. desulfuricans* (PartII/Chapter 1), the partial inhibition observed may be due to the presence of only a *bd*-type enzyme. In fact, the hypothesis that *D. desulfuricans* ATCC 27774 *bd*-type oxygen reductase is also strongly inhibited by cyanide cannot be excluded since the cyanide sensitivity varies significantly among the various *bd*-type enzymes (14). In accordance, no gene for a haem-copper oxygen reductase is detected in the very recently available genome of *D. desulfuricans* ATCC 27774, while both haem-copper and *bd*-type oxygen reductases are encoded by genes present in *D. vulgaris* Hildenborough, *D. desulfuricans* G20, *D. vulgaris* PD4 and *D. vulgaris* Miyasaki. Although all *Desulfovibrio* sp. contain oxygen reductase encoding genes, the role of these enzymes in energy conservation and/or oxygen detoxification remains unclear. Also, the enzymes involved in an electron transport chain that gives electrons to the oxygen reductases of *Desulfovibrio*, constitute an open question and the only example, so far reported, is the *c₅₅₃* that we showed to give electrons to the *cca₃* of *D. vulgaris* Hildenborough.

It was previously shown that in *D. gigas*, a quinol:fumarate oxidoreductase and a *bd*-type oxygen reductase form a membrane-bound electron transfer chain, that couples succinate oxidation to oxygen reduction (13). The analysis of the genomes of *D. desulfuricans* ATCC 27774, *D. desulfuricans* G20, *D. vulgaris* Hildenborough, *D. vulgaris* PD4 and *D. vulgaris* Miyasaki and *D. salexigens* (incomplete genome) reveal the presence of genes for succinate dehydrogenase/fumarate reductases. Furthermore, an ortholog for an alternative respiratory-complex III (*bc₁*

complex) (15, 16) seems to be present in *D. vulgaris* Hildenborough, *D. desulfuricans* G20, *D. vulgaris* PD4 and *D. vulgaris* Miyasaki genomes. The genes encoding this complex are organised in a conserved cluster which also occurs in several other organisms, as for example *Chlorofexus aurantiacus*, *Thermus thermophilus* and *Geobacter metallireducens* (16). Searches in the *D. desulfuricans* ATCC 27774 and *D. salexigens* genomes retrieve similar proteins; however the genes are not present in a conserved gene cluster as in the *Desulfovibrios* mentioned above. The existence of NADH:quinone oxidoreductase (complex I) orthologs in *Desulfovibrio* is not clear from the genome analysis. Although several genes annotated as NADH dehydrogenases are present in some *Desulfovibrio* genomes, the amino acid sequence similarity to complex I subunits is low and not all the subunits seem to exist.

In overall, *Desulfovibrio* have a plethora of systems which play an important role in the survival of these organisms in environments exposed periodically to oxygen and that may allow some of these species to respire oxygen. Until this work, it was thought that *Desulfovibrio* could not growth in the presence of high oxygen concentrations. We have shown that *D. desulfuricans* ATCC 27777 is indeed capable to growth at nearly atmospheric oxygen conditions. Furthermore, the genome analysis revealed that the presence of an extra haem *c* in the haem-copper oxygen reductase subunit II, which we show to occur in *D. vulgaris* Hildenborough, seems to be a feature of *Desulfovibrio* species. Further studies are required to elucidate the molecular basis of this enzyme function.

3 - Tetrapyrrole biosynthesis in *Desulfovibrio*

Desulfovibrios contain different and unusual modified-tetrapyrroles as protein cofactors (17, 18) which are, so far, unique to these organisms. The understanding of their presence requires the study of the tetrapyrrole

biosynthesis of *Desulfovibrio*. With the aim of elucidating the tetrapyrrole biosynthetic pathway in *D. vulgaris* Hildenborough, we have performed the biochemical characterization of several enzymes involved in the conversion of ALA into sirohydrochlorin, namely porphobilinogen synthase, porphobilinogen deaminase, uroporphyrinogen III synthase/methyltransferase and precorrin-2 dehydrogenase and of two cobaltochelatase enzymes (Part II/Chapters 3, 4, 5).

As in the majority of prokaryotes, in *D. vulgaris* Hildenborough, ALA is formed via the C₅ pathway, which involves a glutamyl-tRNA synthetase (encoded by the DVU1693 locus of *D. vulgaris* Hildenborough genome), a glutamyl-tRNA reductase (DVU1461) and glutamate 1-semialdehyde aminotransferase (DVU3168). Two ALA molecules are converted by porphobilinogen synthase (HemB) which, in the case of *D. vulgaris* Hildenborough, we showed to be performed by a zinc dependent-enzyme. This enzyme exhibits conservation of the amino acid residues known to be involved in the binding of the substrate and reaction mechanism. In contrast with the majority of HemBs that form octameric structures, our studies showed that *D. vulgaris* HemB is active in a homo-hexameric form, which is also found in *Rhodobacter* HemB (19). Recently, it was reported that eukaryotic HemB enzymes can undergo rearrangement between octamer and hexamer forms (morphoein forms), with octamer being the form with the highest activity (20, 21).

The porphobilinogen deaminase (HemC) of *D. vulgaris* Hildenborough is very similar to the *E. coli* HemC, regarding spectroscopic and catalytic features (22). Furthermore, the finding of a dipyrromethane cofactor in the catalytic site of *D. vulgaris* HemC supports the essential role of this prosthetic group in the usually observed reaction mechanism. The cofactor binds to the conserved Cys241 of *D. vulgaris*, as judged on the basis of the amino acid sequence comparison with the *E. coli* enzyme (23).

An interesting feature in *D. vulgaris* Hildenborough, is the presence of a bi-functional uroporphyrinogen III methyltransferase/synthase (CobA/HemD) that results from a gene fusion. We showed that the *in vivo* and *in vitro* activities of CobA and HemD are linked to the N-terminal and C-terminal domains, respectively. The *cobA/hemD* gene fusion is present in all available genomes of *Desulfovibrio* sp., as well as in other bacteria such as *Lactobacillus reuteri* or *Clostridium josui*. Interestingly, a serendipitously mutation of Phe446 in *D. vulgaris* CobA/HemD, a residue that is only strictly conserved in *Desulfovibrio* CobA/HemD fusion proteins, to a serine residue was shown to cause impairment of the HemD activity.

The biochemical investigation of the bi-funcional NAD⁺-dependent precorrin-2 dehydrogenase/ferrochelatase (sirohaem synthase, CysG^B) of *D. vulgaris* Hildenborough (PartII/Chapter 3) allowed correction of the protein database annotation, since it was demonstrated that this enzyme is a single NAD⁺-dependent precorrin-2 dehydrogenase (SirC), lacking the proposed chelatase activity. *D. vulgaris* Hildenborough SirC has a consensus NAD⁺ binding motif (GxGxxG_{x10}G) at the start of the N-terminal region, consistent with the NAD⁺-dependent precorrin-2 dehydrogenase activity, which was determined to be significantly higher than the activity reported, for example, for the *B. megaterium* SirC (24). Searches in the *Desulfovibrio* genomes show that all species contain orthologs sharing a significant degree of amino acid sequence identity (43-89 %) to the SirC of *D. vulgaris* Hildenborough. However, an ortholog of a sirohydrochlorin ferrochelatase is absent from all *Desulfovibrio* genomes, an intriguing fact, since these organisms are able to make sirohaem. Also remarkably, is the absence in all the so far available *Desulfovibrio* genomes of orthologs of protoporphyrin IX ferrochelatase, suggesting that *Desulfovibrio* ferrochelatases are distinct from the presently known sirohydrochlorin and protoporphyrin IX ferrochelatases.

We showed that in *D. vulgaris* Hildenborough, sirohydrochlorin can be chelated with either cobalt or iron, by two distinct ATP-independent CbiK cobaltochelataes, one cytoplasmic and one periplasmic, which were named CbiK^C and CbiK^P, respectively (PartII/Chapter 4). When expressed in *E. coli*, the CbiK^P signal peptide is cleaved and the protein is exported to the periplasm. Accordingly, complementation of an *E. coli* mutant strain, unable to perform iron chelation into sirohydrochlorin, is only achieved with a truncated form of CbiK^P that lacks the signal peptide, constituted by the first 28 aminoacids, and therefore remains in the cytoplasm where the ferrochelation of sirohydrochlorin occurs.

CbiK^P is a tetrameric protein with one haem *b* per dimer (Part II/Chapter 4 and 5). The presence of this chromophore in CbiK^P was first suggested from the intense pink/red colour of the protein recombinant fraction and subsequently confirmed by UV-visible, EPR and NMR spectroscopies and later, by structural analysis. Although some protoporphyrin IX ferrochelataes and sirohydrochlorin cobalto- and ferrochelataes contain Fe-S clusters as redox centres (25, 26) (whose function is still unknown), the existence of a haem is, so far, a unique feature within the chelatase family of enzymes. An apo-form of the enzyme, *i.e.* containing no haem, was obtained when the truncated protein $\Delta 28\text{CbiK}^{\text{P}}$ was expressed in the cytoplasm of the *E. coli* cells. Measurements of the specific activity of the haem loaded- and apo- forms of the protein, revealed that the haem is not involved in the sirohydrochlorin metal-chelatase activity. Moreover, the structure of CbiK^P showed that the haem group is located in between two dimers, in a distinct site of the probable porphyrin pocket (27).

The crystal structure of CbiK^P (PartII/Chapter 5) allowed understanding how this protein is structurally organised and how similar is the structure to other available chelatase structures, namely *S. typhimurium* (28) and *Deinococcus radiodurans* (29) CbiKs, *Archaeoglobus*

fulgidus CbiX^S (30) and *Bacillus subtilis* (31), *Saccharomyces cerevisiae* (32) and *Human* (33) protoporphyrin IX ferrochelatases (HemH). More importantly, the structure revealed the location of the haem cofactor and, since this was the first cobaltochelatase successfully co-crystallised with cobalt, also revealed the ligands which are involved in cobalt binding. The protein monomer consists in two domains, A and B, connected by a loop, with a α/β overall topology similar to other members of the chelatase family of enzymes. The haems are located in between two monomers, in a hydrophobic pocket and the haem-iron is coordinated by two symmetrically histidines, namely His96 of domain A of each monomer. The CbiK^P structure co-crystallised with cobalt revealed that domain B contains the ligands that coordinate the cobalt atom namely, His154, Glu184 and His216. A movement of His154 to a location far from the cobalt binding site, observed in the structure lacking cobalt, supports the catalytic role of this residue previously proposed for *S. typhimurium* CbiK (28).

The tetrameric form of *D. vulgaris* CbiK^P is energetically favourable and the assembly with the haem is much more stable than the assembly without the haem. In the tetramer, the four porphyrin pockets, where the chelation reaction occurs and that are accessible from the outside, suggest that the protein may bind and chelate four porphyrin molecules. While the protein fraction of *D. vulgaris* CbiK^P is constituted only by tetramers, CbiK^C protein fraction contains a mixture of tetramers and dimers. A mixture of oligomeric forms were also reported for some cobaltochelatases, such as *Archeoglobus fulgidus* CbiX^S (30) that forms dimers and tetramers and the CbiX^S of *Methanosarcina barkeri* and *Methanobacter thermoautotrophicum*, that form dimers and trimers (34).

The fact that *D. vulgaris* CbiK^P protein is a haem-containing cobaltochelatase rises some questions concerning the function of the haem and the role of a periplasmic sirohydrochlorin chelatase. The fact that i) in *D. vulgaris*, *cbiK^P* gene is located in a putative operon, containing genes for

iron transport proteins; ii) in *Porphyromonas gingivalis* a gene encoding an outer membrane hemin-binding protein, functional as a cobaltochelatease, is also located in a gene cluster encoding iron transporters of the ABC type (35, 36), and iii) in *Dichelobacter nodosus*, the *cbiK* gene and the gene for an orthologue of the periplasmic iron binding protein YfeA, itself a component of an ABC transporter system involved in iron uptake, were shown to be under the control of the ferric uptake regulator (Fur) (37), suggest a possible role of CbiK^P in haem/iron transport in *D. vulgaris*.

Being *D. vulgaris* CbiK^P located in the periplasm, it is not expected to participate in the biosynthesis of vitamin B₁₂. In fact, this process that takes place in the cytoplasmic compartment was shown in this work to also occur in *D. vulgaris* Hildenborough. The presence of a cytoplasmic CbiK^C suggests that this will be the enzyme involved in the production of vitamin B₁₂. In our experiments, both CbiK^C and Δ 28CbiK^P are able to recover the wild type phenotype of a sirohydrochlorin ferrochelatease mutant strain of the same *E. coli*. Previous studies show that the complementation of the *E. coli* mutant strain with cobaltochelateases from other organisms, namely *S. thyphymurim* (38), *Me. barkeri* and *M. thermoautotrophicum* (34) is prevented in the presence of exogenous cobalt. When a different metal ion (nickel) is exogenously added there is no metal-dependent inhibition, suggesting that these cobaltochelateases have more affinity for cobalt than for iron and that nickel does not compete with iron. In the presence of exogenous cobalt, complementation of the sirohydrochlorin ferrochelatease *E. coli* mutant strain with *D. vulgaris* CbiK^C is less efficient than complementation with Δ 28CbiK^P (our unpublished results), suggesting that CbiK^C has higher affinity to cobalt, thus supporting the role of CbiK^C in vitamin B₁₂ synthesis. The presence of genes encoding two putative CbiK cobaltochelateases is, apart from *Desulfovibrio* species, also observed in other genomes, including *Clostridium acetobutylicum*, *Methanobrevibacter smithii* and *Methanospirillum hungatei* but their roles remain to be

investigated. As in *D. vulgaris*, the other available *Desulfovibrio* genomes show that one of the two *cbiK* genes is also encoded in a putative operon containing genes for iron transport proteins. The only exception is observed in *D. desulfuricans* ATCC 27774, where the two *cbiK* genes are not located within an operon. Nevertheless, one of the CbiK enzymes of *D. desulfuricans* ATCC 27774 contains a predicted signal peptide for periplasmic exporting.

4 - Insights on an alternative haem biosynthetic pathway

As previously described in the Introduction's dissertation (Part I/Chapter 3), work performed in the 1990's led to the proposal of an alternative haem biosynthetic pathway in *D. vulgaris* Hildenborough, via precorrin-2 or sirohydrochlorin (39-41). The isolation of a novel intermediate compound, 12,18-didecarboxysirohydrochlorin, an oxidized and decarboxylated form of precorrin-2, from *D. vulgaris* cells, supported the involvement of precorrin-2 or sirohydrochlorin as intermediates in *D. vulgaris* haem synthesis. The proposed alternative pathway (Figure III-2B) takes the following steps: 1) methylation of the C2 and C7 positions of uroporphyrinogen III to form precorrin-2; 2) decarboxylation of the acetic acid chains of rings C and D of precorrin-2 to form 12,18-didecarboxysirohydrochlorin; 3) deacetylation of the acetate groups of ring A and B of the latter intermediate, producing coproporphyrinogen III, which is indistinguishable from the "normal" version (produced directly from uroporphyrinogen III; Figure III-2A).

The authors that proposed the alternative haem pathway showed evidences that *D. vulgaris* soluble fraction contained S-adenosyl-methionine:uroporphyrinogen III methyltransferase and precorrin-2 decarboxylase activities, supporting the first and second steps of the pathway, respectively, but the enzymes responsible for these activities were not purified to homogeneity. Regarding the third step, it remains unclear if

coproporphyrin III and protoporphyrin IX, rather than related compounds, were formed since there were no reproducible experiments that conclusively show the deacetylase activity of the C2 and C7 positions of 12,18-didecarboxysirohydrochlorin.

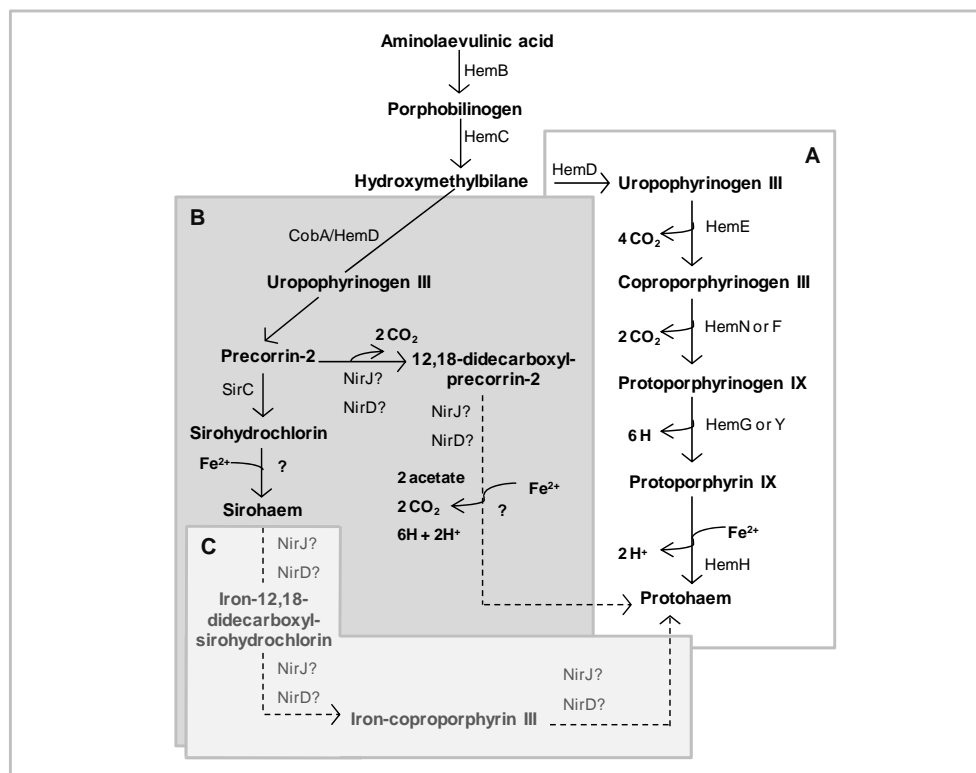


Figure III-2 - Comparison of the general pathway of haem biosynthesis (A) and the alternative pathway (B,C) occurring in *D. vulgaris* Hildenborough, discussed in this thesis. The first steps are common to both pathways. In the alternative pathway, B and C represent two different hypothesis through which haem could be synthesized, both involving ortholog enzymes that occur in haem *dh* biosynthetic pathway.

Our results showed that *D. vulgaris* CobA/HemD is the enzyme responsible for the first step of the alternative pathway, *i.e.*, methylation of the C2 and C7 positions of uroporphyrinogen III to form precorrin-2 (Figure III-2B). The fact that this enzyme is bi-functional suggests that

uroporphyrinogen III is not released as an intermediate, which is in agreement with precorrin-2 or sirohydrochlorin acting as intermediates in the haem biosynthesis. If coproporphyrinogen III is a product formed via precorrin-2, as proposed by Ishida *et al.* (40) then, it would be the substrate of an anaerobic coproporphyrinogen oxidase such as HemN. Interestingly, the *D. vulgaris* genome does encode an orthologue of the radical S-adenosyl-methionine-reliant HemN protein. However, the lacking of a cysteine residue in a conserved motif for the binding of a Fe-S redox group, characteristic of these family of proteins, suggests that *D. vulgaris* protein is a HemN-like protein and proteins lacking this motif have been shown not to be involved in haem biosynthesis (42). Furthermore, genes encoding the other classical enzymes of haem synthesis from uroporphyrinogen III (*hemE*, *hemG* or *hemH*, which encode the enzymes uroporphyrinogen III decarboxylase, protoporphyrinogen IX oxidase and protoporphyrin IX ferrochelatase, respectively) are also absent in all the available *Desulfovibrio* genomes. So, how is haem made? To achieve haem synthesis from precorrin-2/sirohydrochlorin, the acetic acid side chains at C2 and C7 have to be removed. Deacetylation may involve an S-adenosyl-methionine radical mediated reaction (as in precorrin-6A synthase deacetylation reaction) and therefore one radical S-adenosyl-methionine protein could be involved in this step. Interestingly, the genome of *D. vulgaris* encodes one NirD and two NirJ ortholog enzymes, which are involved in the synthesis of haem *d₁*, the cofactor of the nitrite reductase *cdi* found in denitrifier organisms (43, 44), whose corresponding gene, *nirS*, is absent from the *D. vulgaris* genome. Although the role of NirD and NirJ in the biosynthesis of haem *d₁* is not clarified, NirJ is proposed to be radical S-adenosyl-methionine protein. In *Pseudomonas aeruginosa*, NirJ and NirD were hypothesized to be involved in elimination of propionyl side chains and/or in decarboxylation or oxidation reaction (43). However, many NirD proteins, including NirD orthologs of *Desulfovibrio*, are annotated as

transcription factors and the role of NirD remains an open question. Interestingly, in all *Desulfovibrio* genomes, the two *nirJ* genes are located in a putative operon that also contains genes for a NirD and a HemB (prophobilinogen synthase). In *Heliobacillus fasciatus*, NirD is encoded in a putative *nir* operon containing also genes encoding NirL, a homologous protein of NirD, two NirJs and also HemB and HemL (glutamate-1-semialdehyde 2,1-aminotransferase) (45). Studies with *H. fasciatus* NirL showed that the protein is able to bind to a putative promoter, located in the upstream region of the two *nirJ* genes, supporting a role of NirL in the regulation of expression of the *nir* operon (45). Hence, it cannot be excluded that *D. vulgaris* NirD could play a role in regulation of the expression of the two *nirJ* genes. The NirJ enzymes may be one of the missing links in the alternative haem biosynthetic route of *D. vulgaris*, since they could hypothetically act on the removal of the acetic acid chains present in the sirohydrochlorin derived compound and/or could be involved in decarboxylation of the propionate groups to vinyl groups of coproporphyrinogen III (or a derived compound), to form protoporphyrinogen IX (or a derived compound) (Figure III-2B). In our laboratory, investigation is under progress to clarify the role of NirJ and NirD in the *D. vulgaris* haem biosynthetic pathway.

In overall, in *D. vulgaris* Hildenborough, ortholog proteins of the haem *d₁* biosynthetic apparatus may be involved in the transformation of the first branch point of the pathway, which we believe to be precorrin-2, sirohydrochlorin or even sirohaem, into haem. In fact, if we speculate that sirohaem and coproporphyrinogen III are intermediates of the pathway, only three steps would be required to make haem from sirohaem: 1) formation of iron-12,18-didecarboxysirohydrochlorin from sirohaem, 2) formation of iron-coproporphyrin III (the cofactor of the bacterioferritin of *D. desulfuricans* ATCC 27774 (18)) from the latter intermediate, and 3) formation of protohaem from iron-coproporphyrin III (Figure III-2C), by-

passing the two last steps of the classical haem route, the oxidation of protoporphyrinogen IX and ferrochelation of protoporphyrin IX.

Interestingly, in the archaeon *Me. barkeri*, haem biosynthesis was also shown to proceed via a pathway that involves two methylation reactions derived from S-adenosyl-methione (46). Consistent with *Desulfovibrio*, most of the archaeal genomes lack genes encoding ortholog proteins that operate in the classical route that generates haem from uroporphyrinogen III (47), and the gene encoding the nitrite reductase *cd1*, while contain genes encoding NirD and NirJ. Therefore, it was proposed that the alternative haem pathway operative in *Desulfovibrio*, proceeding via precorrin-2 or sirohydrochlorin, could also be the major route in the archaea (46). It is still unknown why some of the *Desulfovibrio* or archaea synthesise haem via an alternative route. It can be speculated that it represents an ancient pathway that was operative when oxygen was not yet present, *i. e.*, before the advent of photosynthesis.

In summary, the work performed in this thesis allow to elucidate for the first time how *Desulfovibrio* performs the initial steps of the tetrapyrrole biosynthetic pathway that generate the compounds from which the various modified tetrapyrroles are synthesized. The biochemical data together with genomic analysis suggests that precorrin-2 or sirohydrochlorin represent the first branch point of the pathway, through which vitamin B₁₂, sirohaem and haem are synthesised.

5 - References

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